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Society of Environmental Toxicology & Chemistry (SETAC)
1101-14th St. N.W., Suite 1100
Washington DC 20005

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9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Dr Walter Kozumbo
AFOSR/NL
110 DUNCAN AVE SUITE B115
BOLLING AFB DC 20332-0001

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ABSTRACT

The metabolic fate of components of creosote, as well as the creosote mixture, was studied in two species of fish, English sole (*Pleuronectes vetulus*) and rainbow trout (*Oncorhynchus mykiss*). Laboratory experiments were conducted to assess the metabolism and DNA adduct formation of aromatic compounds by these fish species. These studies were conducted to determine whether the metabolic pathways of creosote components are similar between fish species which have been shown to be susceptible to hepatotoxic effects of components of creosote. In addition, comparisons of the metabolic products of creosote components formed in live animals and by isolated liver cells were made to determine whether isolated hepatocytes may be used as an alternative to live animals in delineating the mechanisms of metabolism of individual compounds and complex mixtures of xenobiotics. Isolated hepatocytes from English sole and rainbow trout were exposed to either benzo(a)pyrene (BaP), a component of creosote, or a creosote extract and the types of metabolites formed were assessed by reversed-phase liquid chromatography (RPLC) or gas chromatography/mass spectrometry (GC/MS). The types of DNA adducts formed during the metabolism of BaP or the creosote mixture were determined using the [^{32}P]postlabeling assay.

The results showed that BaP was metabolized by English sole and rainbow trout hepatocytes primarily to glucuronide conjugates of hydroxylated BaP derivatives, similar to those detected in bile of English sole exposed to BaP *in vivo*. The major metabolites identified include the 7(8)-hydroxy-8(7)-BaP-β–D-glucopyranosiduronic acid (BaP-7,8-dihydrodiol-glucuronic acid), 1-BaP-β-D-glucopyranosiduronic acid (1-hydroxyBaP-glucuronic acid), and the 3-BaP-β-D-glucopyranosiduronic acid (3-hydroxyBaP-glucuronic acid). Medium containing hepatocytes from both fish species also contained significant amounts of unconjugated BaP-9,10-dihydrodiol. In addition, significant amounts of radioactivity eluted prior to the glucuronide conjugates.

Although the identities of these metabolites are unknown, evidence was obtained to indicate their partial identities as diconjugated metabolites having at least one glucuronic acid moiety. The types of DNA adducts formed by English sole liver and isolated hepatocytes exposed to BaP were identical and corresponded to adducts derived from *anti*-7r,8t-dihydroxy-9t,10t-epoxy-7,8,9,10-tetrahydroBaP (*anti*-BaPDE) and *syn*-7r,8t-dihydroxy-9c,10c-epoxy-7,8,9,10-tetrahydroBaP (*syn*-BaPDE). In contrast, the major DNA adduct formed by rainbow trout isolated hepatocytes exposed to BaP appears to be derived from *anti*-BaPDE. These results demonstrate (A) differences in the metabolism of BaP by English sole and rainbow trout, and (B) that the metabolites produced by hepatic oxidative enzymes cannot predict the types of DNA adducts formed by these fish species.

When English sole were exposed to a complex mixture of creosote components, which was extracted from sediment obtained from a creosote-contaminated estuary in Puget Sound, WA, RPLC and GC/MS analysis of metabolites released by β-glucuronidase hydrolysis of bile revealed the presence of phenolic and dihydrodiol metabolites of dibenzofuran (DBF), ethyl- or dimethyl-alkylated (C_2) DBFs, phenanthrene (PHN), C_2-PHNs, fluorene, C_2 fluorenes, fluoranthene (Flu), pyrene, and BaP. Bile of English sole exposed to the creosote mixture either parenterally (injected into the dorsal sinus cavity) or enterally revealed similar types and proportions of metabolites. English sole hepatocytes, when exposed to the creosote mixture or to the fractionated acidic, basic, or neutral components of the creosote mixture, formed similar metabolites as those detected in bile of English sole. In addition, similar metabolites were also formed by rainbow trout hepatocytes. Analysis of DNA adducts formed in English sole liver and isolated hepatocytes of sole and rainbow trout by the [^{32}P]postlabeling method revealed the presence of similar types of bulky, hydrophobic DNA adducts which chromatographed in the region where the major DNA adducts of BaP, BbF and Flu metabolites chromatograph. The DNA binding of BaP, BbF, and Flu

metabolites were similar in both isolated hepatocytes and liver of English sole exposed to equimolar dosages of these compounds, demonstrating the ability of English sole hepatocytes to correctly predict the *in vivo* genotoxicity of 4-5 ring aromatic hydrocarbons. In addition, the majority of DNA damage resulted from the neutral fraction of the creosote mixture.

These findings demonstrate that both English sole and rainbow trout can efficiently metabolize an individual component of creosote, namely BaP, as well as a creosote mixture to metabolites that are excreted from liver and hepatocytes in the form of glucuronic acid conjugates. However, a fraction of the metabolites formed in hepatocytes became covalently bound to DNA, which may initiate subsequent effects in fish liver. The present results demonstrate the potential usefulness of isolated fish hepatocytes as an alternative to live animals in studies of aquatic toxicology.

FINAL REPORT

ENVIRONMENTAL FATE OF A COMPLEX MIXTURE, CREOSOTE, IN TWO SPECIES OF FISH

Marc Nishimoto

Principal Investigator

April 1994

Cellular Toxicology Group

Environmental Conservation Division

Northwest Fisheries Science Center

National Marine Fisheries Service

2725 Montlake Boulevard East

Seattle, WA 98112-2097

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SUMMARY

The metabolic fate of components of creosote, as well as the creosote mixture, was studied in two species of fish, English sole (*Pleuronectes vetulus*) and rainbow trout (*Oncorhynchus mykiss*). Laboratory experiments were conducted to assess the metabolism and DNA adduct formation of aromatic compounds by these fish species. These studies were conducted to determine whether the metabolic pathways of creosote components are similar between fish species which have been shown to be susceptible to hepatotoxic effects of components of creosote. In addition, comparisons of the metabolic products of creosote components formed in live animals and by isolated liver cells were made to determine whether isolated hepatocytes may be used as an alternative to live animals in delineating the mechanisms of metabolism of individual compounds and complex mixtures of xenobiotics. Isolated hepatocytes from English sole and rainbow trout were exposed to either benzo(a)pyrene (BaP), a component of creosote, or a creosote extract and the types of metabolites formed were assessed by reversed-phase liquid chromatography (RPLC) or gas chromatography/mass spectrometry (GC/MS). The types of DNA adducts formed during the metabolism of BaP or the creosote mixture were determined using the [^{32}P]postlabeling assay.

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INTRODUCTION

Creosote is a complex mixture of mutagenic and carcinogenic compounds and is used primarily as a wood preservative for protection against insects, fungi, and marine borers [1, 2]. Creosote is produced by distillation of coal tar and has been shown to produce a toxic response in benthic communities [3-5]. Contamination of freshwater and marine environments by creosote has been documented, including Eagle Harbor in Puget Sound, Washington [5, 6]. The majority of the contamination resulted from spills at wood treatment facilities or its leaching from treated wood used for marine pilings and foundation timber.

Creosote has been shown to be toxic [7] and two components of creosote, BaP and benz(a)anthracene (BaA), have been shown to be mutagenic upon metabolic activation [8], and carcinogenic in rodent assays [9]. Research on the biotransformation of creosote has been limited because creosote is a complex mixture and methods for the analysis of complex mixtures have not been available until recently [6, 10-14]. Numerous studies have addressed the issue of metabolism of either a component of creosote [i.e., carbazole, BaP] [8, 15, 16] or a model mixture of a class of compounds present in creosote [i.e., azaarenes, polycyclic aromatic hydrocarbons (PAHs)] [17, 18]. Schocket et al. [11, 12] have shown that when creosote was applied to skin of rats, numerous DNA adducts were detected using the [^{32}P]postlabeling assay. The major adducts detected were believed to arise from the aromatic hydrocarbon components of creosote.

Aromatic compounds, such as BaP, require metabolic activation to induce biological effects, and the mechanisms of their metabolism have been studied in mammalian systems both *in vivo* and *in vitro* [9, 19]. Initially, metabolism involves oxidation of the hydrocarbon, usually by the microsomal cytochrome P$_{450}$-dependent enzyme system. Intermediates of substrate-activated oxygen complexes are formed at a number of positions and are relatively unstable metabolites that

undergo three major reactions: (i) rearrangement to form phenols, (ii) hydrolysis catalyzed by the enzyme epoxide hydrolase to form dihydrodiols, and (iii) conjugation with glutathione (GSH) catalyzed by a family of glutathione-S-transferase (GST) isoenzymes. Two major pathways of further metabolism of dihydrodiols and phenols involve conjugation with either glucuronic acid [mediated by uridinediphosphoglucuronosyl transferase (UDPGT)] or with sulfate [mediated by sulfotransferases (ST)] [9, 19]). The GSH, glucuronic acid, and sulfate conjugates are water-soluble and generally considered detoxication products. Unconjugated dihydrodiols and phenols may also be metabolized by further oxidation to form multiple hydroxylated products. During the metabolism of aromatic hydrocarbons, electrophilic intermediates are formed and are capable of binding to DNA, inducing cytotoxicity and mutation in cells in culture, and causing tumors in animals [9, 19]. The carcinogenicity of a series of aromatic hydrocarbons has been found to correlate with the level of covalent binding to DNA in mouse skin [20] and cells in culture [9] and with mutation induction in a cell-mediated assay [9].

Assessing the metabolism of aromatic compounds has been done extensively using microsomal systems, cell culture, tissue culture, and live animals [9, 21-27]. Although the majority of work has been conducted using rodent models, isolated hepatocytes from several species of fish have been used to study the metabolism of genotoxic chemicals [28-31]. Bailey *et al.* [32] have shown that aflatoxin B_1 is metabolised extensively by Mt. Shasta strain rainbow trout (*Oncorhynchus mykiss*) hepatocytes and that the types of DNA adducts formed *in vitro* are similar to those formed *in vivo*. Isolated hepatocytes from brown bullhead (*Ictalurus nebulosus*) [33] and mirror carp (*Cyprinus carpio*, L.) [34] have been used to study the metabolism of BaP and the ability of the metabolites to induce DNA damage. These studies show that isolated hepatocytes of fish are a useful system for delineating the metabolic

pathways of xenobiotics. However, few if any studies have been performed using isolated fish hepatocytes to study the metabolism of a complex mixture, such as crude oil or an organic-extract of contaminated sediment.

Both English sole and rainbow trout have been shown to be susceptible to BaP-induced liver diseases, including hepatocellular carcinoma [35] and preneoplastic foci of cellular alteration [36]. In addition, an organic solvent extract of sediment from a creosote-contaminated estuary, Eagle Harbor in Puget Sound, WA, also induced the foci of cellular alteration in English sole liver [36]. English sole sampled from Eagle Harbor have a relatively high prevalence of liver diseases, including hepatocellular carcinoma [5]. Because these two fish species appear to be susceptible to hepatic diseases induced by aromatic compounds, investigating the metabolism of genotoxic compounds may give insights into the mechanisms of xenobiotic-induced liver tumors.

The proposed study will provide important information regarding the biotransformation of a creosote mixture in isolated hepatocytes of English sole and rainbow trout. In addition, the results will be compared to results obtained *in vivo* when fish are exposed to the creosote mixture. The major goals of this proposal are (A) to determine pathways of the metabolism of creosote and the activation of components present in creosote to DNA adducts by different fish species, and (B) to determine whether fish hepatocytes could be used as a complementary system in understanding the metabolic pathways of xenobiotics present in the marine environment.

SPECIFIC AIMS

The hypothesis to be tested is that isolated hepatocytes of English sole and rainbow trout biotransform components of a complex mixture, such as those present in creosote, in a manner similar to that observed *in vivo* and that the active components of creosote responsible for the majority of DNA adducts are similar to the toxic metabolites identified in mammals. Therefore, to test the hypothesis, isolated hepatocytes of English sole and rainbow trout will be exposed to various fractions and components of a creosote mixture and the creosote mixture itself, in order to determine the biotransformation products of creosote constituents. The creosote mixture used in these studies was an organic solvent extract of sediment collected from Eagle Harbor, WA. Eagle Harbor sediment has been contaminated from creosote spills over several decades, and thus represents an environmentally "weathered" complex mixture containing genotoxic chemicals present in creosote. Comparisons of the types of metabolites and DNA adducts between isolated hepatocytes and liver *in vivo* will provide information on the usefulness of isolated hepatocytes in discerning metabolic pathways of single compounds and complex mixtures.

MATERIALS AND METHODS

Chemicals

The BaP was obtained from Sigma Chemical Co., St. Louis, MO. Carrier free [^{32}P]phosphate (NEX-053) was purchased from NEN Research Products (E. I. DuPont, Wilmington DE). The [^{3}H]BaP and carrier free [^{32}P]ATP (5,000-6,000 Ci/mmol) were purchased from Amersham, Arlington Heights, IL, U.S.A. Both labeled and unlabeled BaP were purified by the method of Varanasi and Gmur [37]. The 7H-dibenzo(c,g)carbazole (DBC) (H0258, purity 99.9%), 7R, 8S, 9S-trihydroxy-10R-(N^2-deoxyguanosyl-3'-phosphate)-7, 8, 9, 10-tetrahydrobenzo[a]pyrene [(+)-*anti*-BaPDE-dGp, AD0884), BaP-9,10-dihydrodiol, BaP-7,8-dihydrodiol, 1-hydroxyBaP, 3-hydroxyBaP, 3-hydroxyBaP-glucuronide, and 3-hydroxyBaP-sulfate were purchased from the Midwest Research Institute, Kansas City, KS. Fluoranthene (Flu), benzo(b)fluoranthene (BbF), benz(a)anthracene (BaA), chrysene (Chr), 1-nitropyrene (1-NP), DBC, and phenanthrene (PHN) were purchased from Aldrich Chemical Company, Milwaukee, WI and were >99% pure. 3-Hydroxyfluoranthene (U 1121) was purchased from Chemsyn Science Laboratories, Lenexa, KS. The 9-fluorenol, 1-pyrenol, 9-phenanthrol, and hexamethylbenzene (internal standard for GC analysis) were provided by Dr. Peggy Krahn of the National Marine Fisheries Service, Seattle, WA.

Tetrabutylammonium bromide (TBAB), EDTA, β-glucuronidase (G-8132), butylated hydroxytoluene (BHT), heparin, Waymouth MB 752/1 medium, bovine serum albumin (A-7030), collagenase (C-9407), nuclease P_1 (N-8630), micrococcal nuclease (N-3755), bicine (B-3876), tris (T-1503), CHES buffer (C-2885), urea (U-1250), spermidine (S-2626), dithiothreitol (D-0632), l-glycerol-3-phosphate (G-7886), adenosine 5'-diphosphate (A-6521), adenosine 5'-triphosphate (A-6144), 2'-deoxyadenosine 3'-monophosphate (D-3014), sodium pyruvate (P-2256), lithium chloride (L-0505), proteinase K (P-6556), spleen phosphodiesterase (P-6897), apyrase

(A-6132), RNase A (R-4875), RNase T_1 (R-8251), RNase T_2 (R-3751), polyethyleneimine in 50% aqueous solution (P-3143) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Bacterial alkaline phosphatase (70035) was purchased from United States Biochemical, Cleveland, OH. Liquified phenol was purchased from either Mallinkrodt, Los Angeles, CA, U.S.A. or from Aldrich Chemical Co., Milwaukee, WI. The solvent vehicle, Emulphor 620™ (polyoxyethylated castor oil), used for administering the BaP and creosote mixture, was a gift from the GAF Corp., New York, NY. Salmon sperm DNA, used as a control for [^{32}P]postlabeling assay, was extracted from testes of mature, laboratory-raised Atlantic salmon (*Salmo salar*) as described below. All other chemicals were analytical grade and used without further purification.

Eagle Harbor Sediment Extract (EHSE)

Extraction of Eagle Harbor Sediment

Sampling of sediment from Eagle Harbor in Puget Sound, WA, was conducted using the NOAA research vessel, *Harold W. Streeter*. Sediment samples (100g) were placed in 1 liter brown bottles, along with 500 g of sodium sulphate and 500 ml methylene chloride. The bottles were sealed with Teflon-lined caps and rolled on a rock-tumbler for 16 hrs at 100 rpm. The methylene chloride was decanted and saved. Another 500 ml of methylene chloride was then added to the bottle and rolled for an additional 6 hrs. The methylene chloride from each extraction was combined and concentrated to 15 ml in an Erlenmeyer flask using a 3-ball Snyder column and a 60°C water bath. The methylene chloride extract was transfered to a Kontes concentrator tube and the volume reduced to 1 ml. The extract was then solvent exchanged by adding 3 mls of acetone and reconcentrating to a final volume of 2 ml.

Chemical Analysis of Eagle Harbor Sediment Extract

The EHSE was passed through an RPLC with two 22.5 x 250 mm stainless steel columns in series, each containing Phenogel 100-Å size-exclusion packing material. The EHSE was eluted with methylene chloride at a flow-rate of 7 ml/minute. The fraction collected corresponded to the region where 3-5 ring aromatic hydrocarbons elute, using biphenyl as an internal standard. The methylene chloride solvent in the collected fraction was exchanged by adding hexane and reducing the volume to 1 ml. An internal standard of hexamethylbenzene was then added prior to analysis by GC.

The cleaned-up EHSE was analysed by a 5970 Hewlett-Packard mass selective detector (MSD), a 59940A Hewlett-Packard HP-UX Chemstation data system, a 5890 Hewlett-Packard GC, and a 7673B autosampler. The EHSE sample was injected splitless onto a DB-5 capillary column, and the split ratio valve opened after 30 seconds with a ratio of 20:1. The oven temperature of 50°C was held for 1 minute, then programmed at 4°C/minute up to 300°C, where the temperature was held for 4 minutes.

Fractionation of Eagle Harbor Sediment Extract

The EHSE was mixed with acetone to give a final concentration of 25 g sediment extracted/ml acetone. The EHSE was fractionated into its neutral, acidic, and basic components using a modified method of Miyashita et al. [38]. To a 0.5 ml aliquot of EHSE was added 0.5 ml diethyl ether. This solution was mixed with 0.5 ml 2N HCl and vortex for 10 seconds. The aqueous phase was removed and placed in a scintillation vial. The organic phase was extracted with 0.5 ml 2N HCl an additional 4 times and the aqueous phases combined. The combined aqueous phase was extracted with diethyl ether and the ether layers combined. The organic phase was then thoroughly extracted with 2N NaOH four times. The ether phase was

dried with sodium sulfate, evaporated under a stream of nitrogen gas, and dissolved in acetone to give the neutral fraction. The soluble components in the HCl fraction were neutralized with 10N NaOH and extracted 5 times with 0.5 ml diethyl ether. The diethyl ether fraction was dried with sodium sulfate and represented the acid-soluble fraction. The soluble components in the NaOH fraction were treated with 12N HCl until the pH was < 2.0. This solution was extracted with diethyl ether 5 times, dried over sodium sulfate, and the solvent removed under nitrogen gas. The residue was dissolved in acetone and represented the base-soluble fraction.

Analysis of the residues showed that, by weight, the neutral fraction represented *ca.* 82%, the acid-soluble fraction *ca.* 11%, and the base-soluble fraction *ca.* 7%, respectively, of the EHSE. The neutral fraction represents primarily unsubstituted and alkylated aromatic hydrocarbons, with the acid-soluble fraction representing aromatic heterocycles (i.e., substituted quinolines, acridines, and pyridines) and the base-solubles representing carboxylic acids, catechols and hydroquinones [38, 39]. The residues of each fraction were dissolved in a volume of acetone representing 2-times the weight of the residue, giving the solution concentrations comparable to the unfractionated EHSE (50 g sediment extracted/ml).

Animals

English sole were caught by otter trawl from Polnell Point, a minimally contaminated site [40] in Puget Sound, Washington. Fish from Polnell Point are characterised by low level contaminant exposure [40] and almost zero prevalence of liver diseases [41]. The fish were fed a diet of minced clams and krill, and held in 4' circular tanks with a constant flow of sea water at 12 ± 2°C before the experiments were started.

The Mt. Shasta strain rainbow trout were a gift from Dr. Mary Arkoosh of the National Marine Fisheries Service in Seattle, WA. Mature female trout (490-520g)

were fed a diet of Purina trout chow and held in 6' circular tanks with a constant flow of fresh water at a temperature of 11 ± 1°C.

Isolation of Hepatocytes

Untreated female English sole and rainbow trout were used for *in vitro* experiments. Hepatocytes were isolated according to previously reported methods [42] with minor modifications. Both Hank's and Waymouth solutions used for English sole hepatocyte isolation contained 176 mM NaCl, where for rainbow trout the concentration was 134 mM. These differences in salt concentration reflected the salt and fresh water environment of English sole and rainbow trout, respectively The liver was perfused with 15 mls of Hank's solution containing 280 units of heparin/ml, using a 30 ml disposable polypropylene syringe and a 23-G needle. The liver was subsequently perfused with Hank's solution containing 0.1 mg collagenase/ml. The liver was minced using 2 single edge razor blades which were previously washed with methylene chloride. The minced liver was gently massaged to dislodge the cells and the hepatocytes were filtered sequentially through two nylon filters (190 and 64 μm mesh). The hepatocytes were recovered by centrifugation at 4°C for 2 min at 200g, washed 3 times with Hank's solution (containing 2 g bovine serum albumin/ml and 14 mg $CaCl_2$/ml), and suspended in Waymouth medium. Cell density was determined using a hemocytometer and cell viability was determined using trypan blue exclusion. Aliquots of hepatocytes were placed into either 50 ml polypropylene tubes or 20 ml glass scintillation vials at concentrations of 8-10 $x10^6$ cells per ml.

Metabolism of Xenobiotics by Isolated Hepatocytes

The [^{3}H]BaP, dissolved in 10 μl acetone, was added at concentrations in the medium of 2 or 40 μM. The concentration of acetone in the medium was 0.03% (v/v). No loss of hepatocyte viability was observed after 60 min or 24 h of incubation at [^{3}H]BaP concentrations of between 2 to 40 μM. Individual xenobiotics were dissolved in acetone and added to the hepatocyte suspension at a concentration

in the medium of 25 μM. The EHSE was added to the hepatocyte suspension in 10 μl aliquots, representing a concentration of 100 mg sediment extracted/ml medium. All vials and tubes were incubated at 17±1°C in a shaker bath under air atmosphere.

Exposure of Animals to Xenobiotics

Benzo(a)pyrene

The [^{3}H]BaP was dissolved in Emulphor 620™-acetone (1:1, v/v) and injected (1 ml vehicle/kg fish) into the dorsal sinus cavity along the dorsal fin of three female English sole at a dosage of 50 mg BaP/kg fish. Control fish (n=3) received vehicle only. After 72 h, the fish were killed and both liver and bile were sampled and stored at -80°C until analysis.

Eagle Harbor Sediment Extract

The Eagle Harbor Sediment Extract (EHSE) was dissolved in Emulphor 620™-acetone (1:1, v/v). English sole (150 ± 30 g) were exposed to EHSE either via injection (1 ml vehicle/kg fish) into the sinus cavity along the dorsal fin, or by gavage. The dosage for both exposures was 25 g sediment extracted/kg fish. Control fish (n=4) received vehicle only via intrasinusoidal injection. After 72 h, the fish were killed and liver and bile were immediately frozen in liquid nitrogen and then stored at -80°C until analyzed.

Analysis of Metabolites

Benzo(a)pyrene Metabolites

Samples of medium and bile were extracted with chloroform, containing 0.0005% BHT as an antioxidant, to remove parent BaP and unconjugated metabolites [43]. The conjugated BaP metabolites present in medium and bile were analysed by ion-pair RPLC [44]. The 3-hydroxyBaP-glucuronide and 3-hydroxyBaP-

sulfate standards, added to each sample, were detected using a Varian Fluorichrome band-filter fluorescence detector (λ_{ex} = 360-380nm; λ_{em} = >420nm) and an on-line Radiomatic Flow-One detector. To determine the BaP metabolite conjugated to glucuronic acid, radioactive peaks were collected and hydrolysed with β-glucuronidase [45-47]. Unconjugated BaP metabolites, along with radioactivity released after β-glucuronidase hydrolysis, were analysed by RPLC. RPLC analyses were performed using a Perkin-Elmer HC ODS/Sil-X 5-μm column (0.26 x 25 cm) [45]. Unconjugated BaP metabolite standards were added to each sample before chromatography. Radioactivity eluting from the column was detected as described above. To confirm the identities of BaP metabolites, samples were reanalysed by RPLC without the addition of BaP metabolite standards. Peaks eluting from the column were collected and analysed by fluorescence spectrophotometry. The excitation and emission spectra of the unknown metabolites were compared to the spectra of known BaP metabolite standards.

EHSE Metabolites

Fluorescence Spectrophotometric Analysis of Bile

Bile samples of English sole exposed to EHSE were mixed with methanol (0.5% v/v) and the fluorescence spectra determined using a Shimadzu RF5000U fluorescence spectrophotometer. The excitation wavelength was set at 200 nm and the emission wavelength was set at 20 nm longer wavelength. Subsequent spectral analyses were conducted with the excitation wavelength increased by 20 nm, with the $\delta(\lambda ex\text{-}\lambda em)$ remaining at 20 nm. The excitation spectrum was then recorded at the maximum emission wavelength. The excitation and emission spectra were then used to determine the wavelength pairs for RPLC analyses of metabolites.

RPLC Analysis of Fluorescent Aromatic Compounds (FACs) in Bile

For the analysis of fluorescence aromatic compounds (FACs) in bile, RPLC analysis of metabolites were conducted using a Waters 715 UltraWISP autosampler, Waters 510 HPLC pumps, and two Perkin Elmer LS-40 fluorescence detectors connected in series. The detectors were set at excitation/emission wavelengths of 290/335 nm [naphthalene standard] and 380/430 nm [BaP standard] to quantitated total fluorescent aromatic compounds in naive bile. Data were obtained using Maxima 820 software.

Metabolites of EHSE Components

Metabolites of components in EHSE conjugated with glucuronic acid were determined by the method of Krahn et al [14]. Aliquots of medium and bile were extracted with chloroform, containing 0.0005% BHT as an antioxidant, to remove parent compounds and unconjugated metabolites [43]. Metabolites remaining in the aqueous phase were incubated with β-glucuronidase to hydrolyse glucuronide conjugates. Metabolites released after 2 hrs of incubation at 37°C were extracted with chloroform. The chloroform was removed under vacuum and the residue was dissolved in methanol. This solution was analysed by RPLC and GC/MS as described below.

Reversed-Phase Liquid Chromatographic Analysis of Metabolites

For the analysis of metabolites of EHSE components and polycyclic aromatic standards, RPLC analysis of metabolites were conducted using a Waters 715 UltraWISP autosampler, Waters 510 HPLC pumps, and a Perkin Elmer LS 4 fluorescence spectrophotometer, a Perkin Elmer 650S fluorescence spectrophotometer, and two Perkin Elmer LS-40 fluorescence detectors connected in series. The detectors were set at excitation/emission wavelengths of 317/385, 355/398, 349/387, and 370/462 nm to quantitate total fluorescent aromatic

metabolites in bile and medium hydrosylates. Data were obtained using Maxima 820 software.

Gas Chromatography-Mass Spectrometry Analyses of Metabolites

The bile and medium extracts were analysed by a 5970 Hewlett-Packard mass selective detector (MSD), a 59940A Hewlett-Packard HP-UX Chemstation data system, a 5890 Hewlett-Packard GC, and a 7673B autosampler. The mass spectrometer was scanned using a sequenced selected ion monitoring (SSIM) descriptor at *ca.* 1 scan/sec. The GC run time was divided into segments in which difference sets of ions were scanned. For full-scan spectra, the mass spectrometer was scanned from 45 to 450 amu at *ca.* 1 scan/sec.

Phenanthrene-d10 was added to samples prior to RPLC clean-up of bile and medium extracts. RPLC clean-up of the samples was necessary to remove biogenic materials in the samples prior to GC analysis [14]. The fraction collected (19-30 minutes) was reduced in volume to 1 ml under nitrogen gas and hexamethylbenzene (GC internal standard) was added. The volume of solution was then further reduced to 30 µl prior to GC-MS analysis.

The concentrations of the metabolites were calculated using single-point response factors and were corrected for the recovery of the surrogate standard. When no commercially available reference standard was available, metabolites were quantitated using a GC/MS response factor for an isomer. As a results, the concentrations determined for some of the metabolites were semiquantitative.

DNA Isolation

Liver and hepatocyte pellets were homogenized in 850 µl of 1% SDS, 10 mM EDTA, pH 8.0, solution containing 20 mg α-amylase, 20 mg RNase A, and 10 units RNase T1. The homogenate was incubated at 37°C for 30 minutes. Proteinase K (0.4

mg in 50 µl of 1% SDS, 10 mM EDTA, pH 8.0, solution) was then added to each sample. The sample was mixed by inverting the tubes, and incubated for an additional hr at 37°C. The homogenate was then extracted with one volume of buffered phenol reagent (454 g phenol, 25 ml *m*-cresol, 1 ml β-mercaptoethanol, 130 ml distilled water, 500 mg 8-hydroxyquinoline, equilibrated with 200 ml 2 M Tris buffer, pH 7.4), which had previously been deoxygenated by bubbling with argon for 30 minutes. After centrifugation at 16,000g for 5 minutes, the aqueous phase was extracted with 1 volume of a phenol:chloroform:*iso*-amyl alcohol (25:24:1, v/v/v) solution. The aqueous phase was then further extracted with CIA (chloroform:*iso*-amyl alcohol, 24:1, v/v) three time to remove any residual phenol. The purified DNA was then precipitated by the addition of 1 volume of -20°C 100% ethanol. If the DNA was sheared, then the mixture was allowed to sit at -20°C for 1 hr. The DNA was pelleted by centrifugation and washed with 70% ethanol. The DNA was then dissolved in 10 mM Tris base/1mM EDTA, pH 7.4, and the concentration estimated by UV spectrophotometric analysis at 260 nm, using an experimentally determined extinction coefficient of 22.9 ml/mg [48]. The purity of DNA was assessed by the determining the ratio of absorbance at 260 and 280 nm. In general, the purified DNA had $A_{260/280}$ ratios of 1.80-1.95.

[^{32}P]Postlabeling Analyses of DNA Adducts

The [^{32}P]postlabeling assay was conducted essentially according to Gupta and Randerath [49], and salmon sperm DNA was used as a negative control in each set of analyses. Briefly, DNA was enzymatically hydrolyzed to deoxyribonucleoside 3'-monophosphates, and nuclease P_1 was used to hydrolyze normal nucleotides to nucleosides, thereby enriching the mixture in adducted 3'-monophosphates [49]. The [^{32}P]labeling was initiated by adding a buffer mix (100 mM bicine, 100 mM magnesium chloride, 100 mM dithiothreitol and 10 mM spermidine, pH 8.75)

containing 100 μCi of [γ-^{32}P]ATP and 8 U of T4-polynucleotide kinase. The [γ-^{32}P]ATP was prepared [49] in the laboratory and the specific activities ranged from 350 to 1540 Ci/mmol.

Polyethyleneimine-cellulose thin-layer chromatography (TLC) of the [^{32}P]labeled adducts [49] was carried out using TLC sheets prepared according to Gupta and Randerath [49]. The solvent systems used in the multi-directional chromatography were as follows: D1- 1.0 M sodium phosphate, pH 6.0; D2 was omitted; D3- 7.65 M urea and 4.32 M lithium formate, pH 3.5; and D4- 7.65 M urea, 1.44 M lithium chloride and 0.45 M Tris, pH 8.0. Elution of the chromatograms in D5 was not done. The [^{32}P]labeled DNA-adducts were located and quantitated using storage phosphor imaging technology. Storage phosphor plates were exposed to the chromatograms for 8 hours and then scanned by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) that recorded location and intensity of radioactivity. The data obtained from the imaging plates were processed with ImageQuant (version 3.0) software (Molecular Dynamics, Sunnyvale, CA) and were then converted to [^{32}P] dpm by calculating a conversion factor through simultaneous exposure of a TLC sheet spotted with a serial dilution of known concentrations of [γ-^{32}P]ATP with each set of sample chromatograms [50, 51]. Total nucleotides were determined by one-dimensional TLC of 5'-labeled nucleotides using 0.24 M ammonium sulfate in 8 mM sodium phosphate, pH 7.4, as a solvent and quantitation of deoxyguanosine 3', 5'-bisphosphate. Adducts levels (nmol adducts/mol nucleotides) were calculated from the storage phosphor readings, the [^{32}P] dpm conversion factor, the specific activity of the [γ-^{32}P]ATP, and the number of total nucleotides analyzed.

Reversed-Phase Liquid Chromatographic Analyses of Oxidative DNA Damage

Analyses of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) was accomplished by RPLC with electrochemical detection [52, 53]. The DNA was dissolved in 0.05M

Tris-HCl buffer, pH 7.5, containing 10 mM EDTA. Ten µl aliquots of the DNA solution were added to 0.5 ml microcentrifuge tubes, which had previously washed with distilled water and methanol. To the DNA solution was added 5 Units of micrococcal nuclease, dissolved in 10 µl of 100 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2. The solution was incubated for 2 hrs at 37°C in a water bath. Ten Units of nuclease P1, dissolved in 10 µl of 30 mM $ZnCl_2$, was then added to the hydrolysis mixture. After an additional 2 hrs of incubation at 37°C, 1 Unit of alkaline phosphatase, dissolved in 10 µl of 0.5 M Tris-HCl buffer, pH 8.0, was added to each sample. The hydrolysis mixture was then incubated at 37°C for an additional 2 hrs. The hydrolysates were then centrifuged for 5 minutes at 16,000xg in an Eppendorf 5415C microcentrifuge. The clear supernatant containing the mononucleosides was then transfered to a limited volume insert vial for RPLC analysis.

Separation of 8-OH-dG was achieved using a Waters 715 UltraWISP autosampler, Waters 510 HPLC pump, and a Waters µBondapak ODS column (10 µm, 3.9 x 300 mm). The 8-OH-dG was separated using a mobile phase of 7.5% methanol in 0.05M sodium phosphate, pH 3.4 [54, 55]. The mobile phase was filtered through a 0.45 µm nylon filter and degassed with helium. The 8-OH-dG was eluted using isocratic conditions, with a flow rate of 2.2 ml/minute and was detected using an ESA Coulochem model 5100A electrochemical detector. The ESA model 5020 guard cell potential was set at +0.48 V and the ESA model 5011 analytical cell potential was set at +0.43 V. Response time was set at 10 seconds. To obtain a steady baseline, the mobile phase was recirculated through the guard and analytical cells at the above potentials. Data were obtained using the Rainin Dynamax data acquisition program, Version 1.2β4.

Statistical Analysis

Comparisons between two means were conducted using Student's t-test. Differences in levels of FACs in bile and DNA adducts in liver of English sole exposed to EHSE were determined using analysis of variance (ANOVA) and Fisher's least protected significance difference test. Multiple and factorial regression analyses were conducted for calibration curves. All statistical analyses were conducted using StatView II or Statview 4.0 (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Experiments were conducted with English sole and rainbow trout. Because of difficulties in assessing the types of metabolites and DNA adducts formed by fish hepatocytes exposed to EHSE, initial studies with BaP were conducted to optimize conditions for analysis of metabolites and adducts. Subsequent studies have utilized GC/MS techniques for the identification of metabolites of components present in creosote, and the [^{32}P]postlabeling assay for the detection of hydrophobic xenobiotic-DNA adducts. These state-of-the-art techniques were instrumental in partially characterizing the types and amounts of metabolites and DNA adducts formed by English sole *in vivo* and *in vitro* and by rainbow trout *in vitro*.

Benzo(a)pyrene

Benzo(a)pyrene is a component of creosote and its metabolism has been extensively studied using fish and rodent models [56]. BaP is carcinogenic in rainbow trout liver [57] and has been shown to induce presumed preneoplastic foci in liver of English sole [36]. Previous studies have reported the formation of several metabolites, including the BaP-7,8-dihydrodiol, BaP-9,10-dihydrodiol, 1-hydroxyBaP, and 3-hydroxyBaP, by English sole and rainbow trout liver enzymes [27, 45, 58, 59]. In addition, the total binding of [^{3}H]BaP-derived radioactivity was also measured in both fish species [27, 37, 46, 47, 58, 60], although adduct characterization was not done. Subsequent studies have identified the major DNA adducts of BaP-exposed English sole as being derived from the *anti*-BaPDE and *syn*-BaPDE [61, 62]. Both *anti*-BaPDE and *syn*-BaPDE are mutagenic in bacterial and mammalian bioassays [9]. The binding of *anti*-BaPDE to plasmid DNA has been shown to activate a c-Ha-*ras1* protooncogene to a transforming oncogene when transfected into NIH3T3 cells [63].

English sole

Metabolism of BaP *in vivo*

Bile from English sole exposed to [^{3}H]BaP via intrasinusoidal injection was analysed by ion-pair RPLC and liquid scintillation counting (Figure 1). Several peaks of radioactivity were detected in different regions of the chromatogram. The identities of the metabolites eluting in Region I were not confirmed because of difficulties in characterizing these conjugates by mass spectrometry. However, ion-pair RPLC analysis of the hydrolysate of β-glucuronidase-treated bile revealed a shift in the retention times of the radioactivity chromatographing in Region I (data not shown).

The glucuronic acid conjugates of BaP metabolites eluting in Region II (Figure 1) were isolated hydrolysed by β-glucuronidase to release the unconjugated BaP metabolites. More than 90% of the radioactivity was extracted into chloroform after β-glucuronidase hydrolysis of peaks 1 and 2. Analysis of the chloroform-soluble metabolites derived from peak 1 by RPLC revealed a single major metabolite having a retention time identical to that of BaP-7,8-dihydrodiol (Figure 2A). Also, two major metabolites derived from peak 2 coeluted with the 1-hydroxyBaP and 3-hydroxyBaP standard (Figure 2B). The fluorescence spectra of these primary BaP metabolites were identical with the spectra of the respective standards. Thus, major identified metabolites formed by English sole liver *in vivo* included the glucuronide conjugates of BaP-7,8-dihydrodiol, 1-hydroxyBaP, and 3-hydroxyBaP.

Region III contained low proportion of radioactivity (Figure 1), indicating that bile does not contain sulfate conjugates of phenolic BaP metabolites.

Metabolism of BaP *in vitro*

Perfusion of English sole liver resulted in the recovery of intact hepatocytes with >95% viability. The metabolism of [^{3}H]BaP by English sole hepatocytes was

linear for up to 60 minutes at a substrate concentration of 40 μM and a cell density of $1x10^6$ cells/ml (Figure 3A). This corresponds to a substrate-to-cell (SC) ratio of 40 nmol [^{3}H]BaP/10^6 cells. The linearity of BaP metabolism by English sole hepatocytes at early time points agrees with previous studies using rat [64, 65], carp [34] and brown bullhead [33] hepatocytes at similar SC ratios. At a substrate concentration of 40 μM BaP, the rate of BaP metabolism by English sole hepatocytes (1330 pmol BaP metabolites/10^6 cells per h) was similar to that of carp (2100 pmol/10^6 cells per h [34]) and brown bullhead (1160 pmol/10^6 cells per h [33]) hepatocytes. At a SC ratio of 2, linearity in BaP metabolism was observed for 20 min for English sole hepatocytes (Figure 3B). Incubating sole hepatocytes with BaP concentrations of 2.5 to 80 μM, gave a K_m value for metabolism of [^{3}H]BaP by English sole hepatocytes of 29 ± 10 μM, which is greater than the K_m of 3.43 ± 0.01 μM determined for BaP metabolism by English sole hepatic microsomes [47]. The curvilinear time-course of BaP metabolism in English sole hepatocytes at 2 μM BaP concentration is consistent with the measured K_m value. The K_m value obtained with English sole hepatocytes is similar to the K_m value of 30 - 40 μM BaP obtained with rat hepatocytes [65]. The higher K_m value for fish hepatocytes compared to fish hepatic microsomes indicates a limitation in the transport of BaP from extracellular medium to the site of metabolism (*e.g.*, cytochrome P-450 in the endoplasmic reticulum) [65].

Analysis of the chloroform-extractable metabolites from medium by RPLC revealed that the BaP-9,10-dihydrodiol was the major unconjugated BaP metabolite present (Figure 4). The identity of the BaP-9,10-dihydrodiol was confirmed by comparing the fluorescence spectra of the unknown metabolite with with the BaP-9,10-dihydrodiol standard. Lesser amounts of phenolic BaP metabolites were present in the medium (Figure 4). No attempt was made to confirm the identities of these metabolites.

English sole hepatocytes extensively metabolised [^{3}H]BaP to conjugated metabolites (Figure 5). The profile of conjugated BaP metabolites was nearly identical with those detected in bile of English sole exposed to [^{3}H]BaP *in vivo* (Figure 1). Analysis of medium by ion-pair RPLC revealed that significant amounts of radioactivity chromatographed in Regions I and II, whereas very small amounts of radioactivity eluted in Region III (Figure 5).

The glucuronic acid conjugates of BaP metabolites eluting in Region II (Figure 5) were isolated from medium and hydrolysed by β-glucuronidase to release the BaP metabolites. More than 90% of the radioactivity was extracted into chloroform after β-glucuronidase hydrolysis of peaks 1 and 2. Analysis of the chloroform-soluble metabolites derived from peak 1 by RPLC revealed a single major metabolite having retention time identical to that of BaP-7,8-dihydrodiol (Figure 6A). Also, two major metabolites derived from peak 2 coeluted with the 1-hydroxyBaP and 3-hydroxyBaP standard (Figure 6B). The fluorescence spectra of these primary BaP metabolites were identical with the spectra of the respective standards. Thus, the major identified metabolites formed by English sole hepatocytes *in vitro* included the unconjugated BaP-9,10-dihydrodiol, the glucuronide conjugates of BaP-7,8-dihydrodiol, 1-hydroxyBaP, and 3-hydroxyBaP.

BaP-DNA Adducts *in vivo*

The binding of BaP metabolites to hepatic DNA was assessed using the [^{32}P]postlabeling assay. English sole were exposed to a dosage of 50 mg BaP/kg fish and the types of DNA adducts in liver was assessed by the [^{32}P]postlabeling assay. The level of total DNA binding in liver was approximately 120 ± 55 (n=3) nmol adducts/mol bases. No DNA adducts were observed in DNA of liver from fish exposed to the solvent vehicle (Figure 7A). The identity of adduct 3 (Figure 7B) is not known. However, adducts 1 and 2 (Figure 7B) have chromatographic properties

similar to the major adducts previously detected in liver of English sole exposed to 100 mg BaP/kg fish [61]. Adduct 1 was attributed to the reaction of *anti*-BaPDE with DNA and adduct 2 was characterized as a *syn*-BaPDE-DNA adduct [61]. Adduct 1 had similar chromatographic properties as the standard *anti*-BaPDE-dGp adduct (Figure 7D). Quantitation of the *anti*-BaPDE-DNA and *syn*-BaPDE-DNA adducts was not possible because of overlapping radioactivities from each adduct.

BaP-DNA Adducts *in vitro*

The types of DNA adducts present in English sole hepatocytes after exposure to 2 μM [^{3}H]BaP were determined after 24 h incubation. [^{32}P]postlabeling analysis revealed the presence of three adducts (Figure 7C) that were absent in hepatocytes not exposed to BaP. The level of total DNA binding in hepatocytes after 24 h incubation was 13 ± 8 nmol adducts/mol bases. The types of BaP-DNA adducts were similar to those observed in liver of English sole exposed to 50 mg BaP/kg fish (Figure 7B), namely the presence of two predominant adducts arising from the the reaction of *anti*-BaPDE and *syn*-BaPDE with DNA.

Summary of Results. Metabolism of BaP by English sole

Metabolites

Previous work has demonstrated that the chemically synthesized GSH conjugates of BaP-4,5-oxide, BaP-7,8-oxide, and *anti*-BaPDE elute in Region I of the chromatogram shown in Figures 1 and 5 [44]. The amenability of metabolites in Region I to hydrolysis by β-glucuronidase indicates the presence of diconjugates with at least one glucuronic acid moiety attached to the BaP metabolites. A recent study has shown that quinol diglucuronides also chromatograph in Region I using similar ion-pair RPLC conditions [66]. The monoglucuronide conjugates of BaP metabolites elute primarily in Region II [44], and the proportion of radioactivity

chromatographing in this region was significantly greater in bile than in medium (Table 1). The glucuronide conjugates of BaP-7,8-dihydrodiol and 3-hydroxyBaP have been detected *in vitro* with cell cultures from carp [34], brown bullhead [67], bluegill sunfish [68, 69], and *in vivo* with English sole [47, 70, 71], and starry flounder [47, 56, 70]. The glucuronide conjugate of BaP-4,5-dihydrodiol is more rapidly excreted from liver into bile of rat than the GSH conjugate of BaP-4,5-oxide [72]. When English sole was exposed to sediment containing [^{3}H]BaP, liver contained a lower proportion of glucuronic acid conjugates than bile [73]. These results [72, 73] support the data in Table 1, indicating that metabolites chromatographing in Region II may be excreted preferentially into bile compared to putative thioether conjugates which chromatograph in Region I (Figure 1).

Similar results showing BaP-9,10-dihydrodiol as the major unconjugated metabolite in medium have been obtained with carp [34] and brown bullhead [33] hepatocytes, and with cell lines of bluegill fry caudal trunk (BF-2 [44, 68, 69]), brown bullhead posterior trunk (BB; [68, 69]) and rainbow trout embryonic gonad (RTG-2 [69]). In agreement with previous studies [45, 47], unconjugated BaP-9,10-dihydrodiol was not detected in bile of sole exposed to [^{3}H]BaP. The presence of the unconjugated BaP-9,10-dihydrodiol in medium of English sole primary hepatocyte cultures is consistent with previous studies indicating that this metabolite is transported in its unconjugated form from liver into extrahepatic tissues, such as edible flesh and ovary [23, 71].

English sole hepatocytes do not form significant amounts of sulfate conjugates of BaP phenols (Figures 5), similar to results using carp [34] and brown bullhead [33] hepatocytes. However, brown bullhead hepatocytes form significantly more sulfate conjugates than carp hepatocytes when BaP-7,8-dihydrodiol was the substrate [33, 34]. In contrast to fish hepatocytes, sulfate conjugates of BaP metabolites are formed in larger amounts by mouse hepatocytes [74]. Differences in

proportion and amount of sulfate conjugates formed by fish and mouse hepatocytes may be related to (a) differences in either the level of the cofactor 3'-phosphoadenosine 5'-phosphosulfate [75], (b) differences in the amount of aryl sulfotransferase and its affinity toward various BaP metabolite substrates [76], or (c) possible inhibition of aryl sulfotransferase by various BaP metabolites may occur in hepatocytes from certain animals [77]. Differences in the types of BaP metabolites, along with amounts of sulfate conjugates, have been noted among various cell types [71]. A fourth factor is that the activity of aryl sulfotransferase isoenzymes may be gender-dependent [78]. Previous studies used hepatocytes from either male mouse [74] or fish of both sexes [33, 34], whereas only female English sole was used in the present study. Studies reporting the formation of sulfate conjugates in fish have not reported gender-dependent differences [71, 79-81].

DNA Adducts

The results demonstrate that the types of DNA adducts are similar in liver of English sole exposed to BaP and BaP-exposed hepatocytes. The major adducts were presumably derived from the *anti*-BaPDE and the *syn*-BaPDE. Adducts derived from both the *anti*-BaPDE and *syn*-BaPDE have previously been detected by fluorescence line-narrowing spectroscopy in hepatic DNA of English sole exposed to 50 and 100 mg BaP/kg fish [62]. Tetrols from both *anti*-BaPDE and *syn*-BaPDE have been detected by RPLC/fluorescence analysis after acid hydrolysis of hepatic DNA from bluegill sunfish [82] and English sole [61] exposed to BaP. In BF-2, RGT-2, and BB cells exposed to [^{3}H]BaP, the *anti*-BaPDE (major adduct) and *syn*-BaPDE have also been detected by RPLC-radiometric analysis [69]. In a previous study, exposure of English sole hepatocytes to [^{3}H]BaP-7,8-dihydrodiol resulted in the formation of the *anti*-BaPDE-dG [28]. However, exposure of hepatocytes to [^{3}H]BaP showed low levels of *anti*-BaPDE-dG adducts, with most radioactivity eluting earlier than the *anti*-

BaPDE-dG standard [28]. Difference in the types of BaP-DNA adducts in English sole hepatocytes, as determined radiometrically [28] or by the [^{32}P]postlabeling assay (Figure 5), may indicate that adducts detected radiometrically by RPLC may not be detected using the current solvent system for the analysis of bulky hydrophibic adducts by [^{32}P]postlabeling. Because the *anti*-BaPDE is considered an ultimate carcinogenic metabolite of BaP [9], the presence of BaPDE-DNA adducts in English sole hepatocytes supports previous results showing the induction of presumptive preneoplastic foci of cellular alteration in English sole exposed to BaP [36].

Rainbow trout

Metabolites of BaP *in vitro*

Isolation of rainbow trout hepatocytes by the two-step perfusion technique yielded an average of $27 \pm 5 \times 10^6$ cells per g liver with a viability of >95%, as assessed by Trypan blue exclusion. No loss in viability was observed after exposure to 10-80 μM [^{3}H]BaP for 90 minutes. In general, the hepatocytes were round and individual.

Trout hepatocytes were incubated with 20 μM [^{3}H]BaP for 30 minutes and the medium was extracted with chloroform. Parent [^{3}H]BaP represented 88 ± 2% of the radioactivity in the organic phase. The major unconjugated metabolite extracted from the medium by chloroform was the BaP-9,10-dihydrodiol (Figure 8). This metabolite represented 44% of the total metabolites extracted in chloroform. In addition, a slight amount of radioactivity (*ca.* 4.5%) chromatographed with the 3-hydroxyBaP standard (Figure 8).

Analysis of conjugated BaP metabolites remaining in media after chloroform extraction by IPLC revealed the presence of four major metabolites (Figure 9). The radioactivity eluting at 4 minutes appears to be due to [^{3}H]H_2O. Very little radioactivity chromatographed with the 3-hydroxyBaP-sulfate standard (Figure 9). A major peak of radioactivity (Figure 9) chromatographed in the region where the

GSH conjugates of BaP oxides and diol epoxides elute [44]. The UV spectrum of metabolite 1 had wavelength maxima of 416, 394, 378, 360, 302, 289, 267 and 257 nm, and the fluorescence spectra had wavelength maxima of 414, 392, 373, 356 and 300 nm for the excitation spectrum and 422 and 449 for the emission spectrum. In addition, no bathochromic shifts in either the excitation and emission spectra were observed when the solution was made basic, indicating that metabolite 1 did not contain a phenolic functional group. When metabolite 1 was incubated with β-glucuronidase, IPLC analysis of the hydrolysate showed that metabolite 1 was amenable to β-glucuronidase treatment. Also, 10-15% of the radioactivity was extracted into the organic phase after β-glucuronidase treatment of purified metabolite 1. Because of difficulties in characterizing metabolite 1 by mass spectrometry, the identity of this conjugated metabolite could not be confirmed.

Analysis of the chloroform-extracted medium by IPLC revealed two major peaks of radioactivity chromatographing in the region where the 3-hydroxyBaP-glucuronide standard elutes (Figure 9). Metabolites 2 and 3 were collected and hydrolysed by β-glucuronidase to release the primary BaP metabolites. Greater than 90% of the radioactivity was extracted into chloroform after β-glucuronidase hydrolysis of metabolites 2 and 3. RPLC analysis of the chloroform-soluble metabolites derived from metabolite 2 revealed that 63% of the total radioactivity chromatographed with the BaP-7,8-dihydrodiol standard (Figure 10A). In addition, two major metabolites derived from metabolite 3 after β-glucuronidase hydrolysis (Figure 10B). Approximately 49% of the radioactivity chromatographed under the 3-hydroxyBaP standard with 18% of the total radioactivity co-eluting with 1-hydroxyBaP (Figure 10B). The fluorescence spectra of these primary BaP metabolites were identical with the spectra of the respective standards.

BaP-DNA Adducts *in vitro*

Rainbow trout hepatocytes were exposed to a concentration of 25 μM BaP for 24 hrs for analysis of DNA adducts. [^{32}P]Postlabeling analysis of trout hepatocyte DNA revealed the presence of a major BaP-DNA adduct (Figure 11B) which was absent in control hepatocytes exposed to vehicle only (Figure 11A). The major adduct had chromatographic retentive properties similar to the (+)-*anti*-BaPDE-dGp standard (Figure 11C). The level of binding of *anti*-BaPDE to DNA was 3.5 ± 0.5 nmol/mol bases.

Summary of Results. Metabolism of BaP by Rainbow trout

Metabolites

Analysis by ion-pair RPLC revealed that glucuronides were the major conjugated metabolites formed by trout hepatocytes, with sulfate conjugates of phenolic BaP metabolites comprising a small proportion of the total metabolites formed. Similar to English sole, rainbow trout hepatocytes do not form significant amounts of sulfate conjugates of BaP metabolites (Figures 5 and 9). Results showing preferred conjugation of 1-naphthol and phenolphthalein with glucuronic acid were obtained with plaice (*Pleuronectes platessa*) isolated hepatocytes [31]. In contrast, significant amount of sulfate conjugates of BaP metabolites were formed by hepatocytes from inbred strains of mice [74]. The glucuronide conjugates detected in medium containing isolated trout hepatocytes exposed to [^{3}H]BaP were derived from the BaP-7,8-dihydrodiol, 1-hydroxyBaP and 3-hydroxyBaP. The glucuronide conjugates of BaP-7,8-dihydrodiol and 3-hydroxyBaP have been detected *in vitro* with cell cultures from English sole (Figure 5, [25]), carp [34], brown bullhead [33, 68], bluegill sunfish [68, 69], and *in vivo* with English sole (Figure 1, [46, 70, 83, 84]) and starry flounder [46, 70, 83]. The BaP-9,10-dihydrodiol was the major unconjugated metabolite present in medium containing trout hepatocytes exposed to [^{3}H]BaP (Figure 8), similar to English sole hepatocytes (Figure 4) In contrast, the

unconjugated metabolites detected in media containing hepatocytes from brown bullhead [33] and toadfish [29] included the BaP-9,10-dihydrodiol and BaP-7,8-dihydrodiol. Also, the BaP-9,10-dihydrodiol, BaP-7,8-dihydrodiol, 9-hydroxyBaP, and 3-hydroxyBaP were present in medium containing rat hepatocytes exposed to [^{3}H]BaP [64]. The presence of unconjugated BaP-9,10-dihydrodiol in extracellular medium is consistent with results showing that BaP-9,10-dihydrodiol is transported to extrahepatic tissues in English sole [85]. The results from this study and other studies [29, 33, 64, 86] indicate that fish may be more efficient than rodents in the UDPGT-catalyzed conjugation of BaP metabolites with glucuronic acid, and that differences among fish species exist in the efficiency of detoxication via glucuronidation of BaP metabolites.

In addition to the glucuronide conjugates, significant amounts of radioactivity chromatographed as a major peak in the region where a GSH conjugate of *anti*-BaPDE elutes (Figure 9). In contrast, English sole hepatocytes form several metabolites eluting in Region I where the *anti*-BaPDE-GSH chromatographs (Figure 5). Because metabolite 1 (Figure 9) was affected by β-glucuronidase-treatment, it appears that at least one glucuronic acid is attached to the unknown BaP metabolite. Diconjugated compounds have been observed in bile of fish [87] and rodents [66, 88]. Thus, it is possible that diconjugated BaP metabolites elute in Region I where GSH conjugates of BaP oxides and diol epoxides chromatograph.

The apparent absence of GSH conjugates in medium containing isolated hepatocytes may indicate that electrophilic metabolites of BaP may not be good substrates for trout hepatic GSH transferase (GST) isoenzymes. In contrast to rats [89], trout liver does not produce a significant amount of GSH conjugates of aflatoxin B_1 (AFB_1) metabolites [90]. Addition of hepatic cytosol and purified GSTs from rainbow trout did not reduce the level of AFB_1-DNA binding after activation of AFB_1 by rat liver microsomes [91]. Relatively high GST activities toward 1-

chloro-2,4-dinitrobenzene (CDNB), but low activity toward *cis*-stilbene oxide, have been measured in trout liver cytosol [92]. Because BaP-GSH conjugates were not detected in significant amounts in extracellular medium containing trout hepatocytes, measurements of the catalytic efficiencies of trout GSTs toward *anti*-BaPDE and other arene oxides are needed to determine the importance of GST enzymes in the detoxication of BaP metabolites by trout hepatocytes.

Another possible explanation for the absence of GSH conjugation of BaP metabolites may be that the level of BaP metabolites present as electrophilic substrates for GSH conjugation are too low for GST-catalyzed conjugation. Although the K_m for CDNB is between 0.4-2 μM for GST enzymes isolated from rainbow trout liver [93], the reported K_m values for *anti*-BaPDE by π-class GST isolated from human placenta and rat kidney are 54 and 14 μM [94, 95]. The rate of biotransformation of [^{3}H]BaP by trout hepatocytes was approximately 35 pmol BaP metabolites formed/10^6 cells/hr. Because arene oxides such as BaP-7,8-oxide and BaP-9,10-oxide are a fraction of the total metabolites formed, the concentration of arene oxides and diol epoxides in the hepatocytes appears to be below the K_m for π-class GST-catalyzed conjugation of [^{3}H]BaP metabolites with GSH. Further studies on the kinetics of GST catalyzed conjugation are required to determine the extent of GSH conjugation in the detoxication of BaP metabolites.

DNA Adducts

In contrast to the types of BaP-DNA adducts formed by rainbow trout hepatic microsomes, where both the *anti*-BaPDE-dG and a BaP phenol-oxide-DNA adduct is formed in roughly the same proportion [96], trout hepatocytes appear to form mainly the *anti*-BaPDE-dG adduct (Figure 11B). Other studies have shown that a BaP phenol oxide-DNA adduct formed by rat hepatic microsomes chromatographs closer to the origin of the the PEI cellulose sheet than the *anti*-BaPDE-dG adduct

under similar chromatographic conditions [10]. Postlabeling analysis of mouse skin, a target tissue of BaP-induced tumorigenesis [97], exposed to BaP results in the detection of mainly the *anti*-BaPDE [98]. The absence of the BaP phenol-oxide-DNA adducts in isolated trout hepatocytes exposed to BaP may indicate that the precursor metabolite of the BaP phenol-oxide is efficiently removed by conjugating enzymes present in isolated trout hepatocytes. Conjugation of the BaP phenol catalyzed by UDPGT is unavailable in microsomal systems because the UDPG acid is absent in microsomal preparations. Studies have shown that the genotoxicity of various carcinogens differs in microsomal systems compared to *in vivo* conditions, such as with isolated hepatocytes [22].

The *anti*-BaPDE has been shown to cause skin cancer in mice and is the most mutagenic metabolite of BaP in bacterial assays [9]. The detection of the *anti*-BaPDE-DNA as the major adduct in trout hepatocytes agrees with the ability of BaP to cause liver cancer in rainbow trout [57]. Although formation of DNA adducts in hepatocytes and eventual formation of liver cancer has been correlated for some genotoxic carcinogens [9], the significance of DNA adducts in the progression of cancer in these fish species remains unknown.

Other Individual Xenobiotics

English sole hepatocytes were exposed under standard conditions to a variety of xenobiotics which are present in EHSE, including BbF and Flu. These compounds have different carcinogenic potentials in rodent models [9, 99-102].

Individual Xenobiotics

Exposure of English sole *in vivo* to BaP, BbF, or Flu resulted in the formation of xenobiotic-DNA adducts (Figure 12). For BbF and Flu, a single major spot comprised the majority of the radioactivity detected. In contrast, two adducts were

observed arising from the activation of BaP. A similar pattern was observed when English sole hepatocytes were exposed to these compounds individually (Figure 13). When BaP, BbF, and Flu were incubated with English sole hepatocytes for 24 hrs, BbF metabolites were bound to hepatocyte DNA at higher levels than either BaP or Flu (Figure 13).

Summary of Results. Other Xenobiotics

The results from these experiments demonstrate that usefulness of English sole hepatocytes in ranking the genotoxic potential of individual xenobiotics, such as BaP, BbF, and Flu, as well as providing information on the mechanism of xenobiotic metabolism. For example, metabolism of BaP by English sole hepatocytes results in the formation of adducts arising from *anti*- and *syn*-BaPDEs. The nature of the BbF and Flu metabolites bound to DNA is unknown; however, studies have shown that the *trans*-9,10-dihydroxy-9,10-dihydro-11,12-epoxy-11,12-dihydroBbF (BbFDE) is not the metabolite responsible for the majority of binding to DNA isolated from rat liver, lung, or peripheral blood [102].

Eagle Harbor Sediment Extract (EHSE)

Work assessing the metabolism of EHSE by English sole and rainbow trout has been difficult due to the numerous chemicals present in the Eagle Harbor sediment extract. The concentrations of selected xenobiotics are presented in Table 2. The major emphasis of the work with EHSE was to determine (a) whether English sole isolated hepatocytes metabolizes a complex mixture of xenobiotics similarly to liver *in vivo*, and (b) if there are species-specific differences in the types of metabolites formed between English sole and rainbow trout hepatocytes. In addition, work on the on the ability of EHSE to induce oxidative stress was also performed to determine ancillary effects of exposure to complex mixtures.

English sole

Metabolism of EHSE *in vivo*

An experiment was conducted to determine whether the route of exposure of creosote in English sole affects the types of metabolites formed in liver and eliminated into bile. In English sole exposed to EHSE via intrasinusoidal injection

or orally, the concentration of bile metabolites, assessed by RPLC at BaP and NPH wavelengths [104, 105], showed no differences between treatments (Figure 14). When bile samples were mixed with methanol at a concentration of 0.5% (v/v) and the fluorescence measured in a Shimadzu RF5000 spectrophotofluorometer at 380 nm, with excitation at 342 nm, a greater concentration of FAC resulting from intrasinusoidal injection compared to oral administration (Figure 15). These wavelengths represent the maximum fluorescence emission of bile from fish exposed to EHSE. Control fish, which were injected intrasinusoidally with vehicle only, had background levels of fluorescence in bile and were significantly lower than exposed animals (Figure 15).

Analysis of metabolites released by β-glucuronidase treatment of bile showed no qualitative differences in the pattern of metabolites between fish exposed to EHSE intrasinusoidally or via gavage (Figures 16A-C). The major contributor to the overall fluorescence detected at several wavelengths was a single metabolite eluting at *ca.* 1000 seconds. The fluorescence spectra of this major metabolite were identical with the excitation and emission spectra of 1-hydroxypyrene.

Analysis of the types of oxidized aromatic hydrocarbons formed by English sole by GC/MS revealed a spectrum of compounds (Figures 17, 18). Because of the complexity of the initial broad-scan analysis of metabolites by GC/MS, quantitation of metabolites were done for only a select number of compounds. These include metabolites of dibenzofuran, C_2 dibenzofurans, fluorene, C_2 fluorenes, phenanthrene, C_2 phenanthrenes, fluoranthene, pyrene, and benzopyrenes. No significant differences in the types and proportions of metabolites were observed in samples from fish exposed to EHSE via different routes except for the benzopyrenols, which were higher in bile of fish exposed to EHSE via intrasinusoidal injection (Table 4). There appears to be a trend towards greater concentration of metabolites arising from 4-5 ring aromatic hydrocarbons in fish

exposed to EHSE via intrasinusoidal injection, although the difference was not significant (paired t-test, $p = 0.1011$).

Metabolism of EHSE *in vitro*

The types of EHSE metabolites and DNA adducts formed by isolated English sole hepatocytes were compared with those formed in English sole exposed to EHSE intrasinusoidally. Fluorescence levels in control bile and medium were much lower than test samples. The pattern of fluorescence and maxima wavelengths were similar for test bile and medium, indicating similar types of metabolites. Test medium was hydrolysed with β-glucuronidase to release the metabolites conjugated with glucuronic acid. The metabolites were subsequently analysed by liquid chromatography with fluorescence detection. Wavelengths for excitation and emission were determined from test bile, using 342 nm for excitation and 380 nm for emission. Chromatographic analysis revealed the presence of a major metabolite of intense fluorescence in samples of test medium which was absent in control medium (Figure 19A, B). This metabolite was collected and its fluorescence spectra compared with bile from English sole exposed to EHSE. The data indicate that this metabolite is the major contributor of the fluorescence present in medium containing isolated sole hepatocytes exposed to EHSE. Comparison with metabolites of other AHs indicates that this metabolite has similar spectral properties as 1-hydroxypyrene. An examination of the other fluorescent peaks eluting from the RPLC column showed no significant differences in the types of metabolites formed by English sole isolated hepatocytes and liver (Figure 19C).

DNA Adducts *in vivo*

English sole were exposed to EHSE either via intrasinusoidal injection or gavage at a dosage of 25 g sediment extracted/kg fish and sampled at 3 days post-

exposure. Analysis of DNA adducts by the [^{32}P]postlabeling assay revealed the presence of numerous radioactive spots having chromatographic properties similar to the (+)-*anti*-BaPDE-dGp standard (Figure 20). Analysis of the autoradiograms revealed similar patterns of DNA adducts in fish exposed to EHSE via instrasinusoidal injection and gavage. Higher levels of DNA adducts were detected in fish exposed to EHSE via intrasinusoidal injection as compared to gavage exposure (Table 5).

Exposure of English sole to EHSE via intrasinusoidal injection resulted in an increase in the levels of 8-OH-dG in hepatic DNA over control values (Figure 21A). When fish were exposed to EHSE via gavage, no statistical differences in levels of 8-OH-dG were observed compared to control or parenterally-exposed fish (Figure 21A).

DNA Adducts *in vitro*

Exposure of English sole hepatocytes to EHSE for 24 hrs at 16 ± 1°C resulted in significant modification of hepatic DNA by bulky metabolites (Figure 22A). The level of adducts [84 ± 54 nmol adducts/mol bases (range of 24-191 nmol/mol bases)] were detected in sole hepatocytes exposed to EHSE, significantly higher than control hepatocytes exposed to solvent vehicle only (Figure 22B). Because the experiments were conducted throughout the year, including periods of spawning, the range of DNA binding probably reflects differences in oxidative enzyme activities [106]. The majority of radioactivity had chromatographic properties similar to the major DNA adducts formed by reaction of the (+)-*anti*-BaPDE-dGp with salmon testes DNA.

Exposure of English sole hepatocytes to EHSE and fractions of EHSE resulted in differential binding of metabolites to DNA (Figure 23). The majority of the binding of EHSE components appear to arise from the neutral fraction of EHSE. Exposure of hepatocytes to either the acidic- and basic-fractions of EHSE resulted in

lower DNA binding levels than either EHSE or the neutral components of EHSE (Figure 23).

Rainbow trout

Studies with rainbow trout were limited to exposure of EHSE to isolated hepatocytes. DNA adducts of genotoxic components of EHSE were detected by the nuclease P_1 enhancement version of the [^{32}P]postlabeling assay.

DNA Adducts *in vitro*

Exposure of rainbow trout hepatocytes to EHSE for 24 hrs at 16 ± 1°C resulted in significant modification of hepatic DNA by bulky metabolites (Figure 24A) compared to Control hepatocytes (Figure 24B). Levels of 150 ± 41 nmol adducts/mol bases were detected in trout hepatocytes exposed to EHSE, significantly higher than for control hepatocytes exposed to solvent vehicle only (4.6 ± 2.4 nmol adducts/mol bases). The majority of radioactivity had chromatographic properties similar to the (+)-*anti*-BaPDE-dGp.

Summary of Results. EHSE

These results demonstrate that regardless of the route of administration, English sole formed similar types of metabolites and DNA adducts. However, differences in the levels of FACs present in bile, as well as the levels of xenobiotic-DNA adducts in liver of English sole were observed (Figure 15, Table 4). Fish have been shown to bioaccumulate lipophilic xenobiotics through their diet [17, 107-112]. Previous studies have shown that individual aromatic compounds, such as pyrene, fluorene, and 2-methylnaphthalene [113], BaP [114-116], and DMBA [117], are poorly absorbed by fish gastrointestinal tract as compared to parenteral routes of exposure. Exposure of English sole to EHSE by gavage is environmentally realistic because the stomach contents of sole caught from Puget Sound have been observed to contain sediment (H.R. Sanborn, personal communication). Bile of English sole exposed to EHSE via gavage had a significantly higher ratio of low-to-high molecular weight aromatic compound derivatives in bile than fish exposed intrasinusoidally (Figure 14). Intramuscular injection of EHSE would result in the distribution of xenobiotics among the various organs, including gills (M. Myers, personal communication). Hence, extrapolation of the results from laboratory studies with fish using parenteral routes of exposure of xenobiotics to field studies where a major route of exposure is enteral should be done with caution.

The metabolism of xenobiotics by intestinal enzymes could change the profile of the types of xenobiotics absorbed by the liver. Xenobiotics have been shown to be metabolized by intestinal cytochrome P-450 of fish [118-126]. In additional, the intestine of various fish species has been shown to possess both glutathione S-transferase [93, 118, 125, 127, 128] and UDPGT [123, 129] activities. Hence, the differences in ratio of high-to-low molecular weight aromatic metabolites, as well as FACs measured at 342/380 nm (Figure 15), may be due to differential uptake of high molecular weight aromatic compounds by the intestine in fish exposed via gavage.

In addition, because the majority of fluorescence in bile of fish exposed to EHSE occurs at wavelengths different from that currently used for monitoring purposes [14, 104, 130], addition of the 342/380 nm pair to monitoring programs may increase the sensitivity of the FAC analysis by selecting the excitation and emission maxima of bile exposed to complex mixtures of xenobiotics containing predominantly aromatic hydrocarbons.

Metabolites from predominant aromatic hydrocarbons present in the EHSE were identified and quantified by GC/MS. As can be seen in Table 3, the ratio of phenanthrols to the PHN dihydrodiol is lower to previously reported values using similar methods [105]. This discrepancy may be attributed mainly to the pretreatment of the inlet column of the GC, although changes in the temperature of the inlet column as well as switching to a larger i.d. inlet column also contributed to the enhanced chromatographic analysis of dihydrodiol metabolites. In addition to the identification of metabolites of PHN, GC/MS analysis of bile hydrosylate demonstrated significant levels of fluoranthenols and benzopyrenols (Table 3). Fluoranthene and BaP are major constituent of EHSE (Table 2) and known mutagens [9]. The levels of BaP metabolites may be overestimated because phenolic metabolites of benzofluoranthenes may also contribute to the detector response. No benzofluroanthene metabolite standards were available for these studies. Hydroxypyrene was one of the major hydroxylated aromatic hydrocarbons detected by GC/MS analysis of the organic solvent-soluble extract released after β-glucuronidase hydrolysis of bile of English sole exposed to EHSE. Because isomeric hydroxypyrenes cochromatograph on the GC column, no quantitation of individual hydroxypyrene metabolites could be made. A previous study had shown that hydroxypyrenes as glucuronide conjugates in bile of English sole sampled from polluted estuaries of Puget Sound [13]. 1-Hydroxypyrene levels in bile of English sole from polluted areas of Puget Sound correlated with the summed concentrations

of selected aromatic hydrocarbons in bile [13]. Studies have shown that 1-hydroxypyrene is the major PAH metabolite in urine of rats exposed via diet to a coal tar [131]. Other studies have shown that the 1-hydroxypyrene may be used as a chemical dosimeter of exposure to PAHs [132-134]. Pyrene has been detected in extracts of contaminated sediments sampled throughout US marine waters [135]. The present study showing hydroxypyrenes as major metabolites detected in bile of English sole exposed to a complex mixture of xenobiotics provide further support for the use of hydroxypyrene as a chemical monitor of exposure sentinel animals to PAHs of anthropogenic origin.

As noted with the studies of BaP, the types of metabolites formed by English sole upon exposure to EHSE is not predictive of the types of adducts and level of DNA binding. Analysis of bile of English sole exposed to EHSE and medium containing English sole hepatocytes exposed to EHSE (Figures 16, 19; Table 3) revealed similar types of metabolites. When fish were exposed to EHSE via different routes, [^{32}P]postlabeling analysis revealed similar DNA adduct patterns but different levels of DNA binding *in vivo*. In contrast to *in vivo* experiments, [^{32}P]postlabeling analysis of DNA from hepatocytes exposed to EHSE *in vitro* revealed a different pattern of adducts. The identities of the major DNA adducts formed upon exposure to EHSE are unknown. However, the chromatographic properties of these adducts indicate that the xenobiotic moieties are consistent with bulky, hydrophobic-type structures similar to BaP and BbF. These differences in adduct profile may be the results from several factors, including (a) differences in the uptake of xenobiotics from the blood into hepatocytes *in vivo* compared to direct exposure *in vitro*, as well as differences in dosage of EHSE components *in vivo* compared to *in vitro*, and (b) contribution of DNA adducts from other cell types in liver (i.e., biliary cells, peripheral red blood cells, pancreatic cells) compared to predominantly hepatocytes *in vitro*. Studies with mammals have shown that

differential DNA adduct profiles are related the dose of carcinogen [136]. Kinetic considerations are important in determining binding of xenobiotics where the concentration of toxicants are below K_m. It has been shown that low K_m pathways of metabolism is reponsible for the decreased level of O^6-methylguanine in rats exposed to nicotine-derived nitrosoketone [137]. In addition, synergistic and antagonistic effects have been observed in the metabolism of complex mixtures [138-140]. It has been shown that 9-hydroxyBaP inhibits the metabolism of BaP be English sole liver microsomes [47]. These results suggest that differences in the concentration of xenobiotics in hepatocytes *in vitro* and *in vivo* may alter the DNA adduct profiles. Another possible explanation for the differences in DNA adduct profile between liver and isolated hepatocytes may be the contribution of DNA adducts from other cell types [141-143]. The DNA isolated from excised liver contain nucleic acids from hepatocytes as well as other cell types present in liver, such as peripheral blood, biliary and pancreatic cells, and macrophages. In contrast, [^{32}P]postlabeling analysis of hepatocytes involves mainly DNA from hepatocytes. Although the contribution of DNA adducts from other cell types in liver of English sole has not been delineated, other cell types could be a source of DNA adducts which are quantitated by the [^{32}P]postlabeling assay. Additionally, whereas DNA repair enzymes could affect the DNA adduct profile [144], recent studies have shown that quantitative but not qualitative changes in DNA adduct profile were observed in English sole exposed to EHSE from 4 hrs to 84 days [51]. Although more work is needed to determine the factors involved in the binding of metabolites to DNA, the ability to rank the genotoxic potential of complex mixtures (i.e., EHSE and its components in the neutral, acidic, and basic fractions, Figure 23) makes this system attractive in complementing *in vivo* genotoxicity assays.

Both English sole and rainbow trout hepatocytes metabolized EHSE components to form similar DNA adducts, although minor differences were

observed in the pattern of adducts. The level of binding of EHSE components to DNA was not significantly different between species, similar to the results with BaP. Both fish species appear to be susceptible to BaP-induced liver cancer [35, 36]. The present results demonstrate the applicability of fish hepatocytes to study the genotoxicity of BaP and a complex mixture. However, similar studies should be performed using hepatocytes from fish of the same age used in tumorigenesis studies. Age-dependent changes in enzyme levels, activities, and isoenzyme composition have been reported [145-149]. Studies involving hepatocytes from younger fish may be more appropriate in understanding the mechanisms of xenobiotic metabolism during tumor initiation.

The increase in levels of 8-OH-dG in liver of English sole exposed to EHSE via intrasinusoidal exposure (Figure 21) indicate that oxidative stress occurred in English sole. Using GC/MS-SIM, the levels of 8-hydroxyguanine (8-OH-Gua) were reported for English sole from sites in Puget Sound, Washington, and from Newport, Oregon [150, 151]. Substantially higher levels of oxidized nucleic acid were observed in these studies [150, 151] compared to the results from the present report (Figure 21). The difference in levels of oxidized nucleic acids reported between studies may be due to differences in methods used for the detection and quantitation of oxidative DNA damage, as well as the analysis of deoxyribonucleoside adducts done in this study compared with the detection of oxidized nucleic acid bases [150]. A recent report noted that the higher level of 5-hydroxycytosine and 5-hydroxyuracil obtained by GC/MS compared to RPLC-ECD could be due to artifactual oxidation of DNA during acid hydrolysis or during derivatization of samples for GC/MS analysis [152]. In addition, oxidized bases, such as 8-OH-Gua, may arise from either DNA or RNA. A recent report showed approximately a 10-fold greater excretion rate of 8-OH-Gua than 8-OH-dG in urine of rats fed a nucleic acid-free diet, and an 18-fold greater concentration of 8-OH-Gua than 8-OH-dG in spent media which contained

Escherichia coli [153]. Oxidative RNA damage has been detected in liver of Sprague Dawley rat exposed to 2-nitropropane [154]. We have used RNase A, T1, and T2 to minimize the amount of RNA contamination during isolation of DNA prior to analysis by [^{32}P]postlabeling [50] and RPLC with electrochemical detection. Use of only RNase A during the isolation of English sole DNA can result in 20-50% contamination, on a molar basis, of the final "purified" DNA (W.L. Reichert, personal communication). Thus, detection and quantitation of 8-OH-dG gives a more accurate determination of the extent of oxidative DNA damage than analysis of oxidized nucleic acid bases. Studies with mammals have suggested that the promotional activity of certain chemicals may be related to their ability to induce oxidative DNA damage [155, 156]. It has been demonstrated that English sole exposed to contaminated sediment extracts, or residing in contaminated sediments, have elevated levels of bulky, hydrophobic DNA adducts in liver [40, 51, 157]. Thus, co-occurrence of both bulky DNA adducts and hydroxylated DNA bases in liver of English sole may be important in the etiology of hepatocarcinogenesis in this fish species.

CONCLUSION

The results of this research provide information on the metabolism and genotoxicity of a complex mixture of chemical contaminants present in creosote. Creosote contamination is prevalent in coastal waterways, especially in marinas and waterways utilizing wood pilings treated with creosote. The genotoxicity of creosote components indicates that diseases, such as liver cancer, may arise in animals that are exposed to creosote components.

The results from the experiments described herein show similarities in the metabolism and activation of BaP and creosote components by English sole and rainbow trout. Also, the similarities in the types of metabolites and DNA adducts formed in liver and isolated hepatocytes indicates that isolated fish hepatocytes may be used as an alternative to live animals in studies of mechanisms of metabolic activation of genotoxic compounds present in complex mixtures. In addition, the results with fish hepatocytes may elucidate some of the important factors operating *in vivo* in determining the genotoxic potential of co-exposed xenobiotics. Identification of DNA adducts are important in understanding the bioavailability, and hence biological activity, of complex mixtures of sediment-associated contaminants on the health of benthic organisms. These studies provide a base of information for future studies on the fate and effect of creosote contaminants on marine organisms.

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Table 1. Proportion of conjugated benzo(a)pyrene metabolites formed in English sole *in vivo* and in primary hepatocyte cultures *in vitro*[a].

Region[b]	Bile (n=3)	Medium (n=3)
I	55 ± 4.4[c]	65 ± 4.0
II	39 ± 3.7[c]	27 ± 3.8
III	5.4 ± 2.4	7.6 ± 1.0

[a] Female English sole were exposed intramuscularly to 50 mg [^{3}H]BaP/kg fish and killed 3 days later. Hepatocytes were isolated from female English sole and incubated with 2 μM [^{3}H]BaP for 24 h.

[b] The conjugated benzo(a)pyrene metabolites were separated by ion-pair RPLC [44]. The proportions of radioactivity were determined in the three regions, which were assigned according to where (I) GSH conjugates, (II) glucuronide conjugates, and (III) the 3-hydroxybenzo(a)pyrene-sulphate standards elute ([44], Figure 3).

[c] Significantly different from corresponding values for medium. The percent radioactivity chromatographing in each region was arcsine-transformed and differences between bile and medium were analysed by paired t-test [158].

Table 2. Concentrations of predominant aromatic hydrocarbons in Eagle Harbor sediment extract.

Contaminant	Concentration (ng/g wet weight)
2-ring AHs	
Naphthalene	56,000
2-Methylnaphthalene	17,000
1-Methylnaphthalene	380,000
Biphenyl	110,000
2,6-Dimethylnaphthalene	180,000
3-ring AHs	
Acenaphthylene	8,800
Acenaphthalene	840,000
2,3,5-Trimethylnaphthalene	56,000
Fluorene	920,000
Phenanthrene	1,900,000
Anthracene	320,000
1-Methylphenanthrene	110,000
4-ring AHs	
Fluoranthene	1,200,000
Pyrene	730,000
Benz(a)anthracene	180,000
Chrysene	150,000
Benzo(b)fluoranthene	50,000
Benzo(j/k)fluoranthene	44,000
5/6-ring AHs	
Benzo(e)pyrene	26,000
Benzo(a)pyrene	34,000
Perylene	7,900
Indeno[1,2,3-cd]pyrene	9,600
Dibenz(a,h)anthracene	2,800
Benzo(ghi)perylene	6,700
Σ Selected Aromatic Contaminants	7,338,800
% recovery of	
naphthalene-d8	72
acenaphthene-d10	79
benzo(a)pyrene-d10	89

Table 3. Concentrations (ng/µl bile, ± sem) by GC/MS of selected metabolites of predominant aromatic hydrocarbons in bile of English sole exposed to Eagle Harbor sediment extract via different routes.

Metabolite	Intrasinusoidal	Gavage
Dibenzofuranol	4.17 ± 1.51	9.06 ± 3.21
Dibenzofuranol dihydrodiol	0.108 ± 0.059	0.532 ± 0.121
C_2 Dibenzofuranols	6.78 ± 1.64	6.62 ± 1.06
Fluorenols	21.6 ± 8.67	39.8 ± 7.37
9-Fluorenol	1.01 ± 0.367	1.32 ± 0.306
C_2 Fluorenols	3.94 ± 0.77	3.32 ± 0.614
Phenanthrols	34.1 ± 11.8	38.9 ± 6.07
9-Phenanthrol	0.955 ± 0.367	1.257 ± 0.245
Phenanthrene dihydrodiol	34.9 ± 13.4	44.2 ± 6.42
C_2 Phenanthrols	7.45 ± 1.12	6.11 ± 1.48
Fluoranthenols	52.6 ± 10.2	41.2 ± 6.04
3-Fluoranthenol	35.6 ± 9.74	23.3 ± 4.15
1-Pyrenol	55.1 ± 13.0	35.5 ± 5.91
Benzopyrenols[a]	50.4 ± 11.1	12.1 ± 4.54
3-Benzopyrenol	1.95 ± 0.415	0.273 ± 0.214
Benzopyrene dihydrodiol	0.225 ± .090	not detected
% recovery of		
naphthalene-d8	72	
acenaphthene-d10	79	
benzo(a)pyrene-d10	89	

[a] Values for intrasinusoidal injection were significantly higher than values for gavage exposure ($p < 0.05$) as assessed by ANOVA and Fisher's least significant difference test [158]. Because other metabolites of 5-ring aromatic hydrocarbons, such as benzofluoranthenes, may contribute to the detector response, the difference in concentrations of benzopyrenols are considered tentative.

Table 4. Levels of xenobiotic-DNA adducts formed by English sole exposed to Eagle Harbor sediment extract via different routes.

Route of exposure	nmol adducts/mol bases
Vehicle (control)	2.25 ± 1.31
Intrasinusoidal	213 ± 6.61[a]
Gavage	16.0 ± 8.74

[a] Significantly different from values for control and gavage treatment ($p < 0.0001$) as assessed by ANOVA and Fisher's least significant difference test [158].

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FIGURE CAPTIONS

1. Ion-pair reversed-phase liquid chromatographic analysis of conjugated BaP metabolites present in bile of English sole exposed intramuscularly to 50 mg [^{3}H]BaP/kg fish. Unconjugated BaP metabolites were extracted with chloroform and conjugated BaP metabolites in the aqueous phase were concentrated using a Sep-Pak cartridge (Waters, Division of Millipore) and eluted with methanol before RPLC analysis. Radioactivity was quantified using a Radiomatic Flow-one detector. The three regions were assigned according to where (I) glutathione conjugates, (II) glucuronide conjugates, and (III) the sulphate standards elute [44]. The standards 3-hydroxyBaP-glucuronic acid (3-GA) and 3-hydroxyBaP-sulphate (3-S) were added to samples prior to chromatography and monitored by fluorescence detection. Major fractions of radioactivity 1 and 2 eluting in Region II were collected for further analyses.
2. Reversed-phase liquid chromatography analysis of radioactivity released after β-glucuronidase hydrolysis of fractions 1 and 2, which were detected by ion-pair reversed-phase liquid chromatographic analysis of bile of English sole exposed to [^{3}H]benzo(a)pyrene (Figure 1). Fractions 1 and 2 (depicted in Figure 1) were collected, concentrated on a Sep-Pak cartridge (Waters, Division of Millipore) and eluted with methanol. Fractions 1 and 2 were hydrolysed with β-glucuronidase and the released radioactivity was analysed by RPLC. Unlabeled BaP metabolite standards were added to the sample before analysis. Radioactivity was quantified using a Radiomatic Flow-one detector.
3. Time-course of benzo(a)pyrene (BaP) metabolism by isolated English sole hepatocytes. Hepatocytes were incubated with either (A) 40 μM [^{3}H]BaP/ml medium, or (B) 2 μM [^{3}H]BaP/ml medium. Aliquots of medium + cells were removed at specified intervals. The cells were pelleted by centrifugation and unmetabolized [^{3}H]BaP was extracted from the medium by 0.15 M potassium

hydroxide:hexane partitioning [159]. Radioactivity remaining in the aqueous phase was quantified by liquid scintillation spectrometry. For Figure A, $r = 0.98$; $p < 0.001$.

4. Chromatogram of unconjugated BaP metabolites present in medium after incubation of English sole hepatocytes with 2 μM [^{3}H]BaP. The metabolites were extracted with chloroform [43] and analysed by RPLC. Unlabeled BaP metabolite standards were added to the sample before analysis. Radioactivity was quantified using a Radiomatic Flow-one detector.
5. Ion-pair reversed-phase liquid chromatographic analysis of conjugated BaP metabolites present in medium containing isolated English sole hepatocytes and [^{3}H]BaP. Unconjugated BaP metabolites were extracted with chloroform and conjugated BaP metabolites in the aqueous phase were concentrated using a Sep-Pak cartridge (Waters, Division of Millipore) and eluted with methanol before RPLC analysis. Radioactivity was quantified using a Radiomatic Flow-one detector. The three regions were assigned according to where (I) glutathione conjugates, (II) glucuronide conjugates, and (III) the sulphate standards elute [44]. The standards 3-hydroxyBaP-glucuronic acid (3-GA) and 3-hydroxyBaP-sulphate (3-S) were added to samples prior to chromatography and monitored by fluorescence detection. Major fractions of radioactivity 1 and 2 eluting in Region II were collected for further analyses.
6. Reversed-phase liquid chromatographic analysis of radioactivity released after β-glucuronidase hydrolysis of predominant [^{3}H]benzo(a)pyrene metabolites detected by ion-pair reversed-phase liquid chromatographic analysis of medium containing English sole hepatocytes (Figure 5). Fractions 1 and 2 (depicted in Figure 5) were collected, concentrated on a Sep-Pak cartridge (Waters, Division of Millipore) and eluted with methanol. Fractions I and II were hydrolysed with β-glucuronidase, the released radioactivity was extracted

with chloroform and analysed by RPLC. Unlabeled BaP metabolite standards were added to the sample before analysis. Radioactivity was quantified using a Radiomatic Flow-one detector.

7. Autoradiograms of thin-layer chromatogram profiles of [^{32}P]postlabeled DNA. For intact liver, English sole were exposed to either vehicle only (A) or to 50 mg [^{3}H]BaP/kg fish (B) for 3 days. Isolated hepatocytes from English sole were exposed to 2 μM [^{3}H]BaP (C) for 24 hours. DNA was isolated and analysed using the nuclease P_1 version of the [^{32}P]postlabeling technique. The *anti*-BaPDE-dGp standard was labeled to give a chromatography standard (D).
8. Reversed-phase liquid chromatography chromatogram of unconjugated [^{3}H]benzo(a)pyrene metabolites present in medium after incubation of rainbow trout hepatocytes with 2 μM [^{3}H]BaP. The metabolites were extracted with chloroform [43] and analysed by RPLC. Unlabeled BaP metabolite standards were added to the sample before analysis. Radioactivity was quantified using a Radiomatic Flow-one detector.
9. Ion-pair reversed-phase liquid chromatographic analysis of conjugated [^{3}H]benzo(a)pyrene metabolites present in medium containing isolated rainbow trout hepatocytes. Unconjugated BaP metabolites were extracted with chloroform and conjugated BaP metabolites in the aqueous phase were concentrated using a Sep-Pak cartridge (Waters, Division of Millipore) and eluted with methanol before RPLC analysis. Radioactivity was quantified using a Radiomatic Flow-one detector. The three regions were assigned according to where (I) glutathione conjugates, (II) glucuronide conjugates, and (III) the sulphate standards elute [44]. The standards 3-hydroxyBaP-glucuronic acid (3-GA) and 3-hydroxyBaP-sulphate (3-S) were added to samples prior to chromatography and monitored by fluorescence detection. Major fractions of

radioactivity (peaks 1 and 2) eluting in Region II were collected for further analyses.

10. Reversed-phase liquid chromatographic analysis of radioactivity released after β-glucuronidase hydrolysis of predominant [^{3}H]benzo(a)pyrene metabolites detected by ion-pair RPLC analysis of medium containing rainbow trout hepatocytes (Figure 9). Fractions 1 and 2 were collected, concentrated on a Sep-Pak cartridge (Waters, Division of Millipore) and eluted with methanol. Fractions 1 and 2 were hydrolysed with β-glucuronidase, the released radioactivity was extracted with chloroform and analysed by RPLC. Unlabeled BaP metabolite standards were added to the sample before analysis. Radioactivity was quantified using a Radiomatic Flow-one detector.
11. Autoradiograms of thin-layer chromatograms of [^{32}P]postlabeled DNA isolated from rainbow trout hepatocytes. Isolated hepatocytes from trout were exposed to acetone only (A) or to 25 μM [^{3}H]BaP (B) for 24 hours. DNA was isolated and analysed using the nuclease P_1 version of the [^{32}P]postlabeling technique. The (+)-*anti*-BaPDE-dGp standard was analysed to determine relative chromatographic retentive properties (C).
12. Autoradiograms of DNA adducts formed by English sole hepatocytes exposed to solvent vehicle (A), 25 μM benzo(a)pyrene (B), 25 μM fluoranthene (C), or 25 μM benzo(b)fluoranthene (D) for 24 hrs. Reactions were run in 20 ml scintillation vials placed in a constant temperature water bath held at 17°C. The DNA were isolated and analysed using the nuclease P_1 version of the [^{32}P]postlabeling technique.
13. DNA binding levels of benzo(a)pyrene (BaP), benzo(b)fluoranthene (BbF), and fluoranthene (Flu) metabolites to English sole hepatic DNA *in vivo* and *in vitro*. English sole hepatocytes were incubated for 24 hrs at 17°C with 10 μM of each aromatic hydrocarbon, or with acetone as a control. English sole were also

exposed intramuscularly to each compound separately for 72 hrs at a dosage of 100 μmol/kg fish.

14. Levels of fluorescent aromatic compounds in bile of English sole exposed intrasinusoidally or via gavage to 25 g Eagle Harbor sediment extract/kg fish for 3 days. Bile samples were analysed directly by reversed-phase liquid chromatography and the total fluorescence monitored at two different wavelength pairs. Fluorescence was normalized to either (A) naphthalene (NPH; λ_{ex}=290 nm, λ_{em}=335 nm), or (B) benzo(a)pyrene (BaP; λ_{ex}=380 nm, λ_{em}=420 nm). FAC_{NPH} and FAC_{BaP} levels for bile of fish exposed either intrasinusoidally or via gavage were significantly different from control values. Exposure of English sole to Eagle Harbor sediment extract either intrasinusoidally or via gavage did not result in differences between FAC_{NPH} values and FAC_{BaP} values.
15. Spectrophotofluorometric analysis of fluorescent aromatic compounds in bile of English sole exposed intrasinusoidally or via gavage to 25 g Eagle Harbor sediment extract/kg fish for 3 days. Bile samples were diluted with methanol to a concentration of 0.5% bile in methanol (v/v) and the fluorescence measured in a Shimadzu RF5000 spectrophotofluorometer at 380 nm, with excitation at 342 nm. The FAC levels for bile of fish exposed either intrasinusoidally or via gavage were significantly different from control values ($p<0.001$). Bile of English sole to Eagle Harbor sediment extract intrasinusoidally has significantly higher levels of FACs ($p<0.001$) than bile of fish exposed via gavage. All values were analysed by ANOVA and Fisher's least significant difference test [158].
16. Reversed-phase liquid chromatographic analysis of fluorescent metabolites formed by English sole exposed to Eagle Harbor sediment extract. Metabolites were derived from bile of English sole exposed either intrasinusoidally (A) or

via gavage (B) to 25 g Eagle Harbor sediment extract/kg fish for 3 days. Control fish receive vehicle only. Water-soluble metabolites were hydrolysed with β-glucuronidase for 2 hrs and primary metabolites were extracted into chloroform. Samples were injected onto a Water HPLC system and metabolites were separated using a 0-100% methanol in water gradient. The metabolites were detected by a Perkin Elmer LS-40 fluorescence detector, programmed at an excitation wavelength of 342 nm and an emission wavelength of 380 nm. The scale was expanded (C) to show the pattern of metabolites having lower fluorescence.

17. Gas chromatographic/mass spectrometric analysis of metabolites of dibenzofuran, C_2 dibenzofurans, fluorene, and C_2 fluorenes released after β-glucuronidase hydrolysis of bile of English sole exposed via gavage to Eagle Harbor sediment extract. The metabolites were analysed by a 5970 Hewlett-Packard mass selective detector (MSD), a 59940A Hewlett-Packard HP-UX Chemstation data system, a 5890 Hewlett-Packard GC, and a 7673B autosampler. The mass spectrometer was scanned using a sequenced selected ion monitoring descriptor at *ca.* 1 scan/sec. The GC run time was divided into segments in which difference sets of ions were scanned for (A) Dibenzofuranols, (B) C_2 Dibenzofuranols, (C) Fluorenols, (D) C_2 Fluorenols.

18. Gas chromatographic/mass spectrometric analysis of metabolites of phenanthrene, C_2 phenanthrenes, fluoranthene/pyrene, and benzopyrenes released after β-glucuronidase hydrolysis of bile of English sole exposed via gavage to Eagle Harbor sediment extract. The metabolites were analysed by a 5970 Hewlett-Packard mass selective detector (MSD), a 59940A Hewlett-Packard HP-UX Chemstation data system, a 5890 Hewlett-Packard GC, and a 7673B autosampler. The mass spectrometer was scanned using a sequenced selected ion monitoring descriptor at *ca.* 1 scan/sec. The GC run time was divided into

segments in which difference sets of ions were scanned for (A) Phenanthrols, (B) C_2 Phenanthrols, (C) Fluoranthenols/Pyrenols, (D) Benzopyrenols.

19. Reversed-phase liquid chromatographic analysis of fluorescent metabolites formed by English sole hepatocytes exposed to Eagle Harbor sediment extract. Metabolites were derived from medium containing English sole hepatocytes exposed to Eagle Harbor sediment extract (A). A comparison of the fluorescence response for test and control medium is presented in (B), where the control sample represent medium containing English sole hepatocytes exposed to acetone only. Comparison of metabolites formed by English sole hepatocytes and liver *in vivo* is presented in (C), where fish were exposed intrasinusoidally for 3 days. For all *in vitro* experiments, hepatocytes were exposed to Eagle Harbor sediment extract for 24 hrs at 17°C. Water-soluble metabolites were hydrolysed with β-glucuronidase for 2 hrs and primary metabolites were extracted into chloroform. Samples were injected onto a Water HPLC system and metabolites were separated using a 0-100% methanol in water gradient. The metabolites were detected by a Perkin Elmer LS-40 fluorescence detector, programmed at an excitation wavelength of 342 nm and an emission wavelength of 380 nm.

20. Autoradiograms of thin-layer chromatogram profiles of [^{32}P]postlabeled DNA isolated from liver of English sole exposed to Eagle Harbor sediment extract via different routes. English sole were exposed to acetone only (A) or to a dosage of 25 g sediment extracted/kg fish for 3 days either intrasinusoidally (B), or via gavage (C). DNA was isolated and analysed using the nuclease P_1 version of the [^{32}P]postlabeling technique.

21. Levels of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in liver of English sole exposed to Eagle Harbor sediment extract via different routes. Fish were exposed either intrasinusoidally or via gavage and sacrificed 3 days post-

exposure. The DNA was isolated and the level of 8-OH-dG was estimated using RPLC with electrochemical detection (A). A representative chromatogram of the standard 8-OH-dG is presented in (B), whereas the calibration curve for estimating the amount of 8-OH-dG in samples is presented in (C). Values for intrasinusoidal injection of EHSE was signicantly higher ($p<0.05$) than control values, as assessed by ANOVA and Fisher's least significant difference test [158].

22. Autoradiograms of thin-layer chromatogram profiles of [^{32}P]postlabeled DNA isolated from English sole hepatocytes exposed to Eagle Harbor sediment extract. Isolated hepatocytes from English sole were exposed to acetone only (A) or to 10 µl of a 50 g sediment extracted/ml solution of Eagle Harbor sediment extract (B) for 24 hours. DNA was isolated and analysed using the nuclease P_1 version of the [^{32}P]postlabeling technique.

23. DNA binding of Eagle Harbor sediment extract (EHSE) and fractions of EHSE in English sole hepatocytes. Isolated hepatocytes from English sole were exposed to acetone only (control) or to 10 µl of a 50 g sediment extracted/ml solution of EHSE for 24 hours. In addition, hepatocytes were exposed to the neutral, acidic, or basic fractions of EHSE at concentrations similar to that of the original EHSE solution (see *Materials and Methods*). DNA was isolated and analysed using the nuclease P_1 version of the [^{32}P]postlabeling technique.

24. Autoradiograms of thin-layer chromatogram profiles of [^{32}P]postlabeled DNA isolated from rainbow trout hepatocytes incubated for 24 hrs at 17°C with acetone only (A) or to 10 µl of a 50 g sediment extracted/ml solution of Eagle Harbor sediment extract (B). The DNA were isolated and analysed using the nuclease P1 version of the [^{32}P]postlabeling technique.

Figure 1

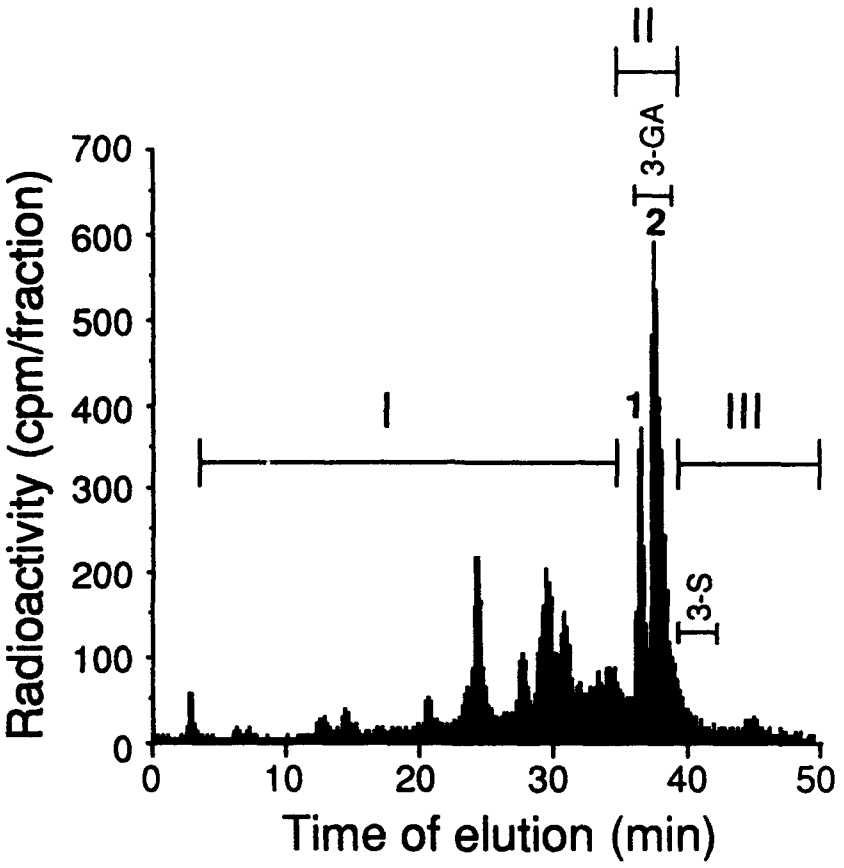
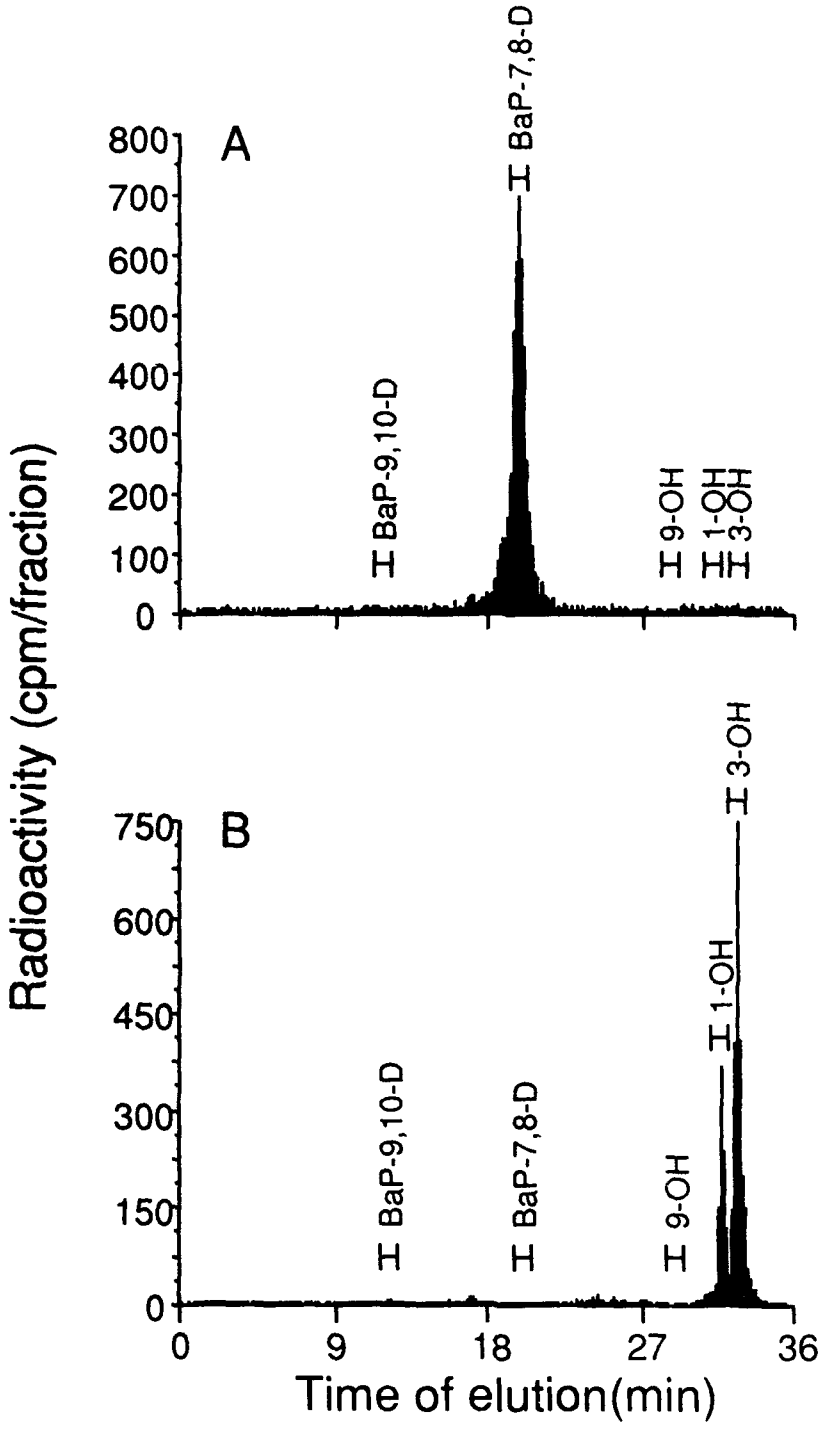

II
3-GA
2
I
1
III
3-S
Radioactivity (cpm/fraction)
700
600
500
400
300
200
100
0
0
10
20
30
40
50
Time of elution (min)

Figure 2


A
B
BaP-9,10-D
BaP-7,8-D
9-OH
1-OH
3-OH
Radioactivity (cpm/fraction)
Time of elution(min)
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100
200
300
400
500
600
700
800
150
450
750
9
18
27
36

Figure 3

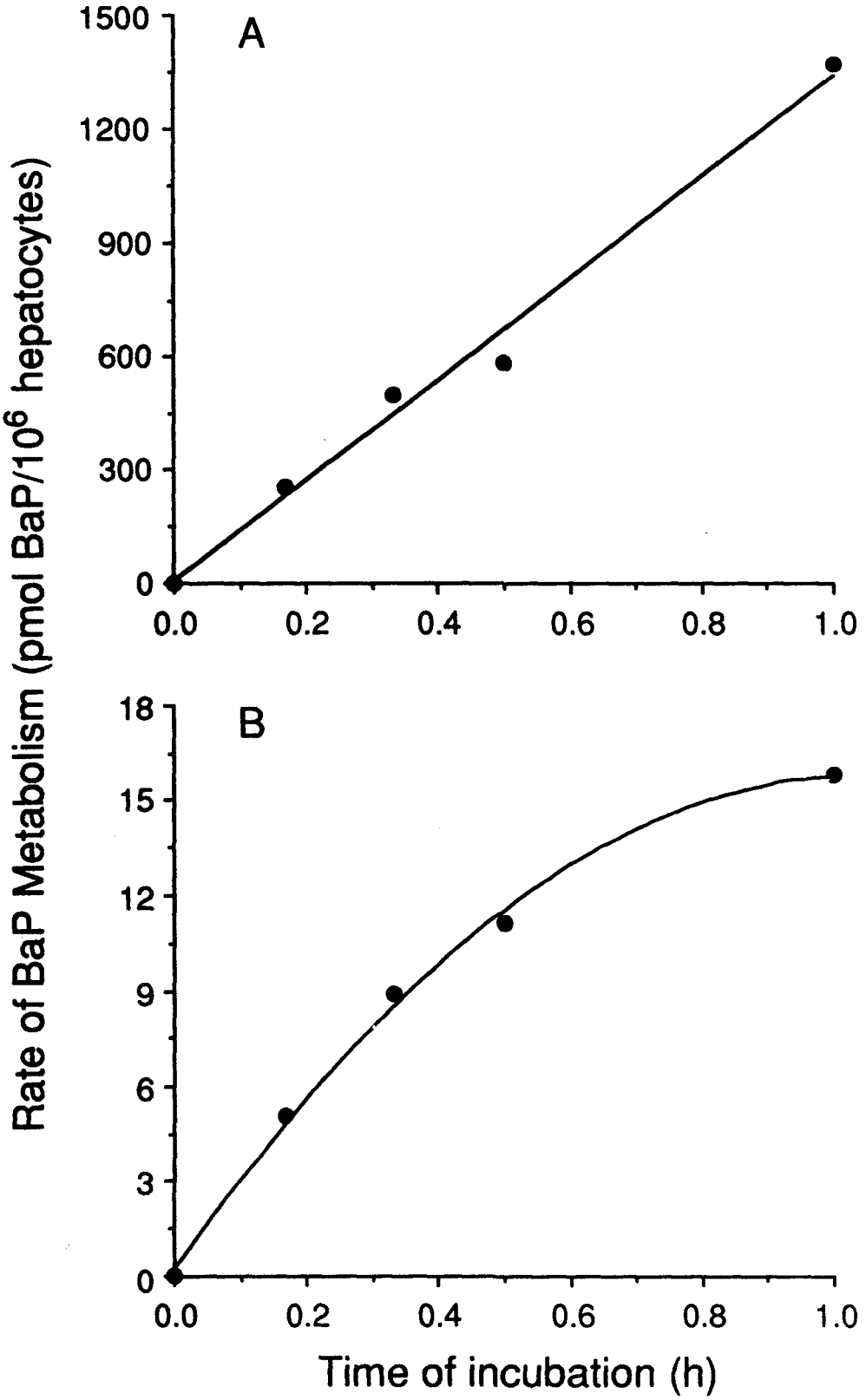

A
B
1500
1200
900
600
300
0
18
15
12
9
6
3
0
0.0
0.2
0.4
0.6
0.8
1.0
Rate of BaP Metabolism (pmol BaP/10^6 hepatocytes)
Time of incubation (h)

Figure 4

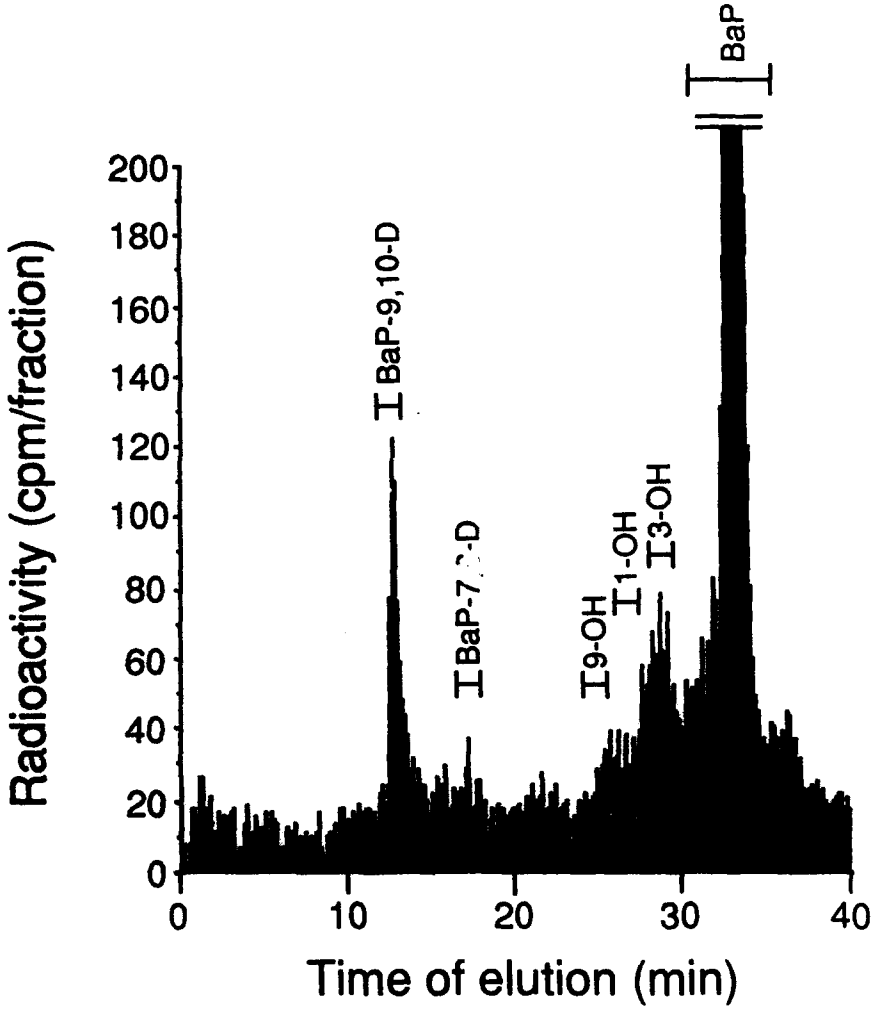

BaP
BaP-9,10-D
BaP-7,8-D
9-OH
1-OH
3-OH
Radioactivity (cpm/fraction)
200
180
160
140
120
100
80
60
40
20
0
0
10
20
30
40
Time of elution (min)

Figure 5

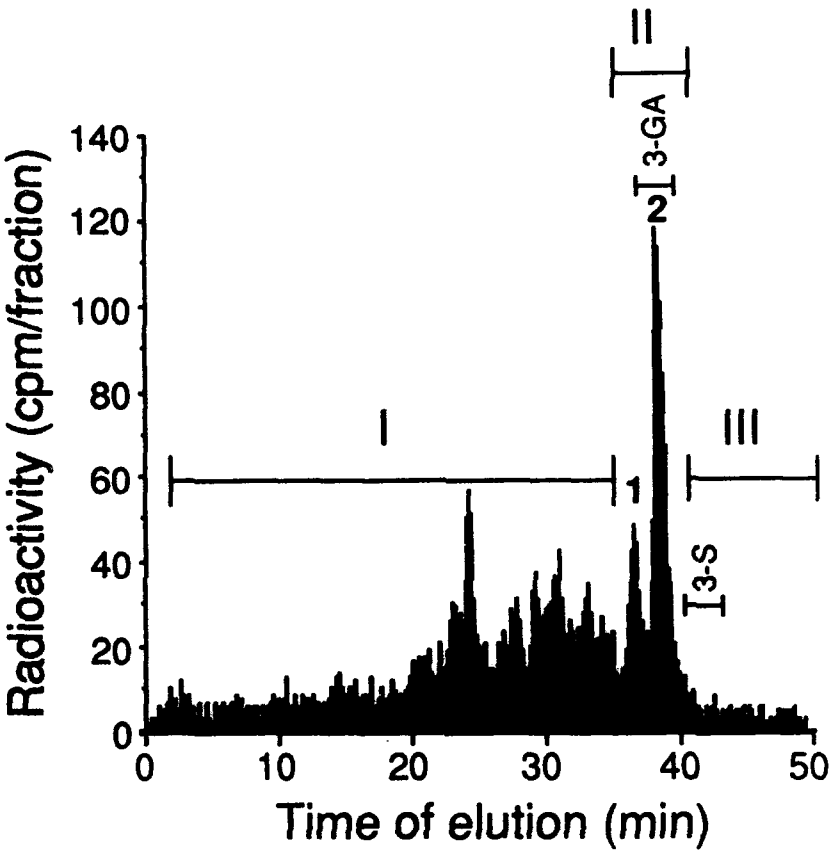

Radioactivity (cpm/fraction)
140
120
100
80
60
40
20
0
0
10
20
30
40
50
Time of elution (min)
I
II
III
1
2
3-GA
3-S

Figure 6

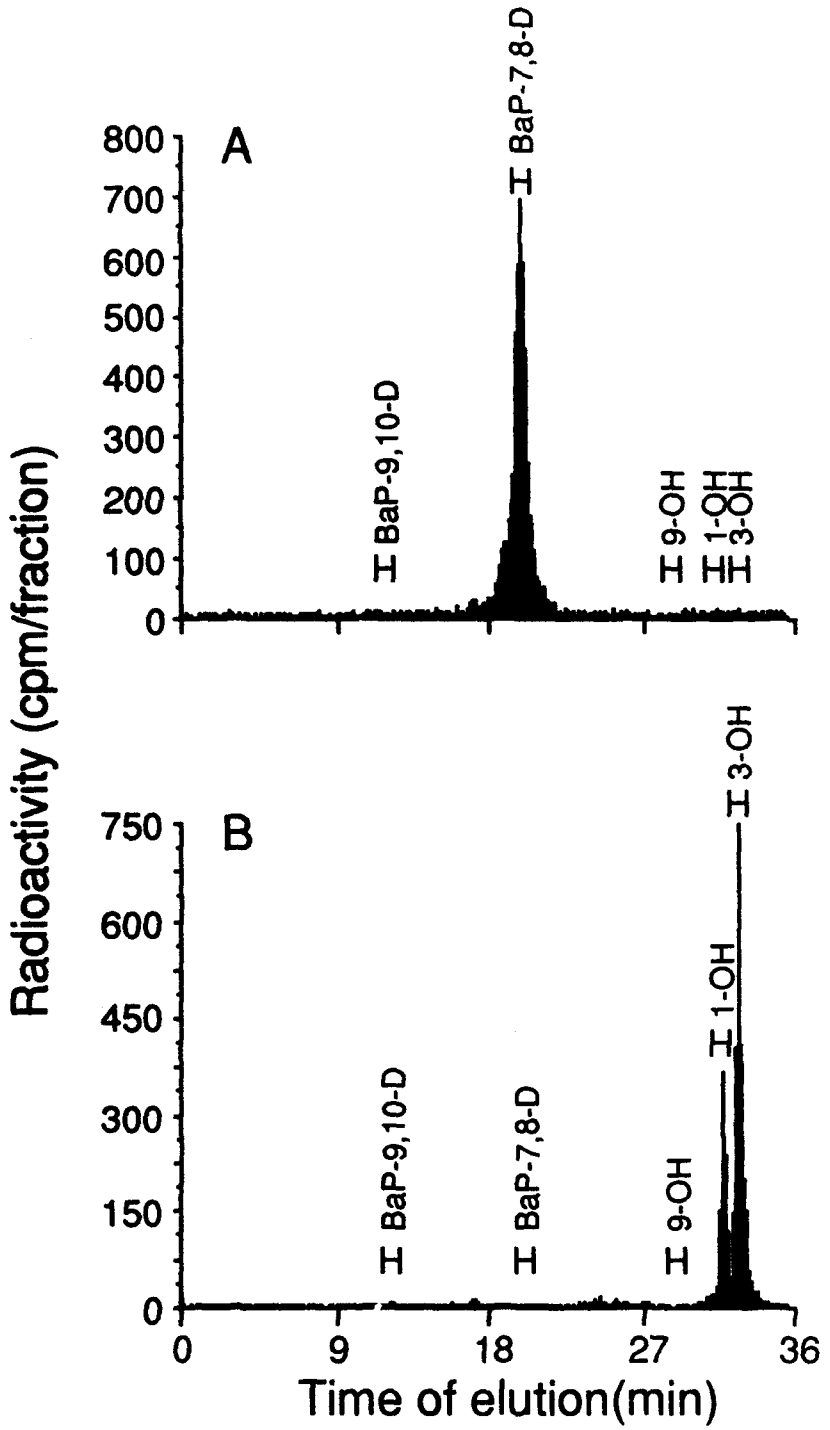

A
B
BaP-9,10-D
BaP-7,8-D
9-OH
1-OH
3-OH
Radioactivity (cpm/fraction)
Time of elution(min)
800
700
600
500
400
300
200
100
0
750
600
450
300
150
0
0
9
18
27
36

Figure 7

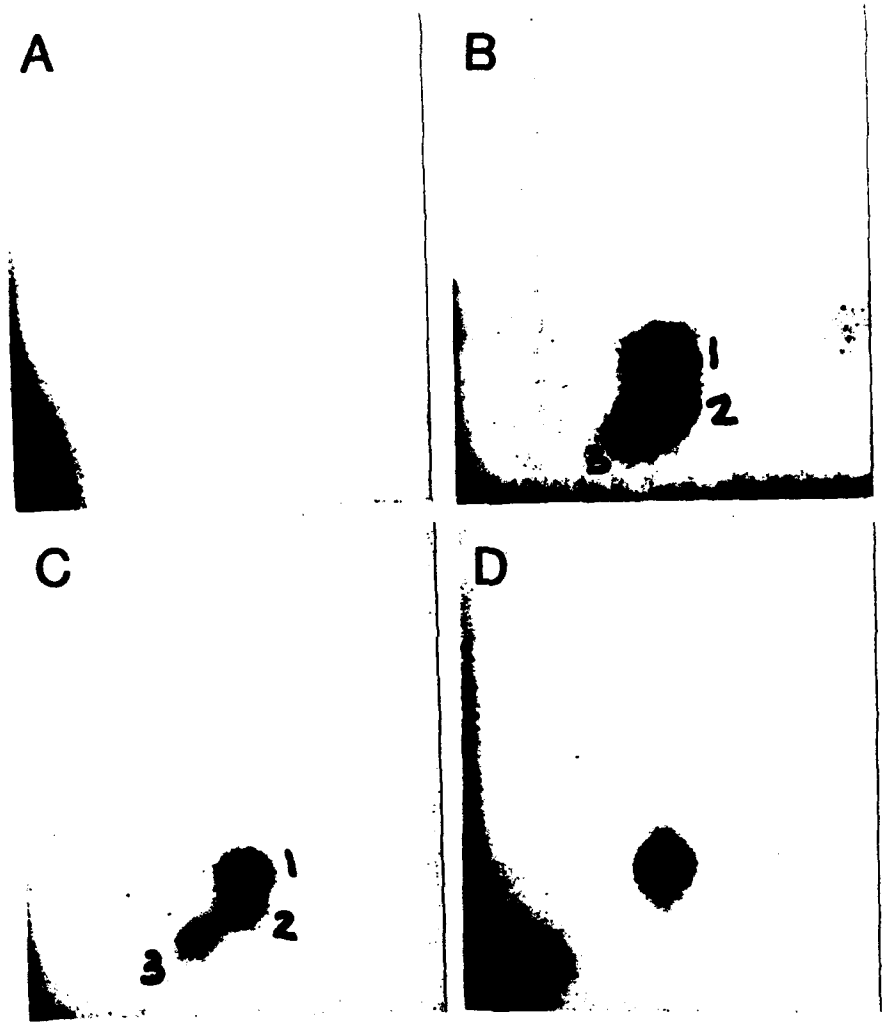


Figure 8

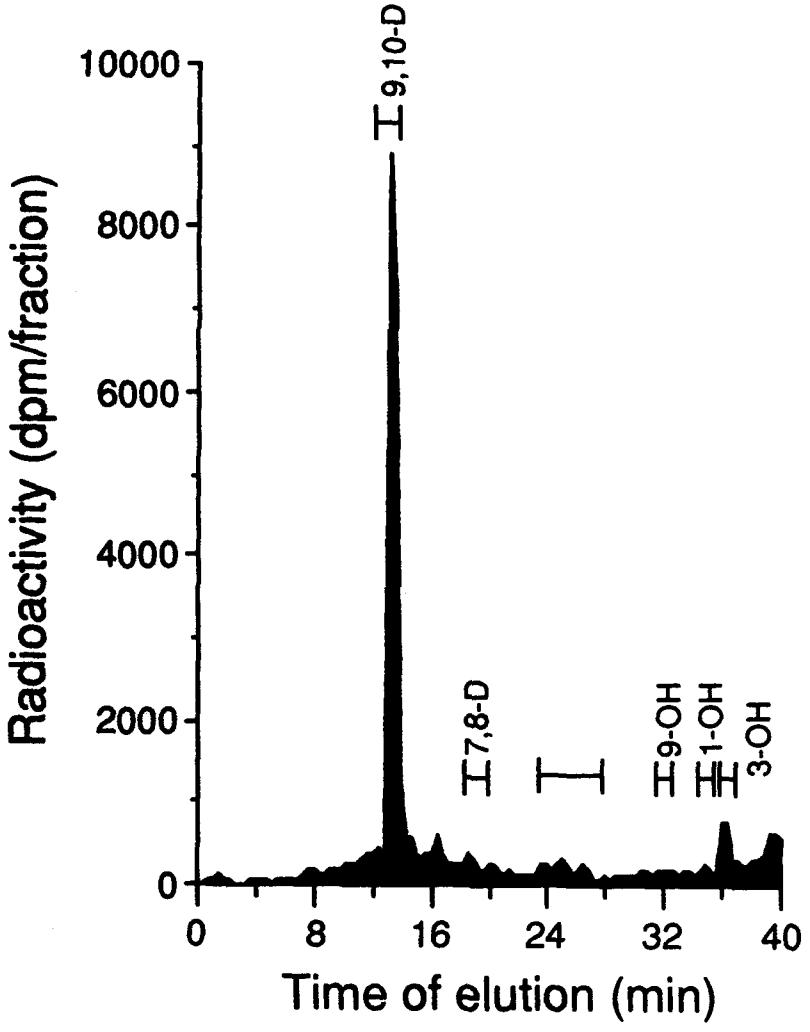

10000
8000
6000
4000
2000
0
Radioactivity (dpm/fraction)
9,10-D
7,8-D
9-OH
1-OH
3-OH
0
8
16
24
32
40
Time of elution (min)

Figure 9

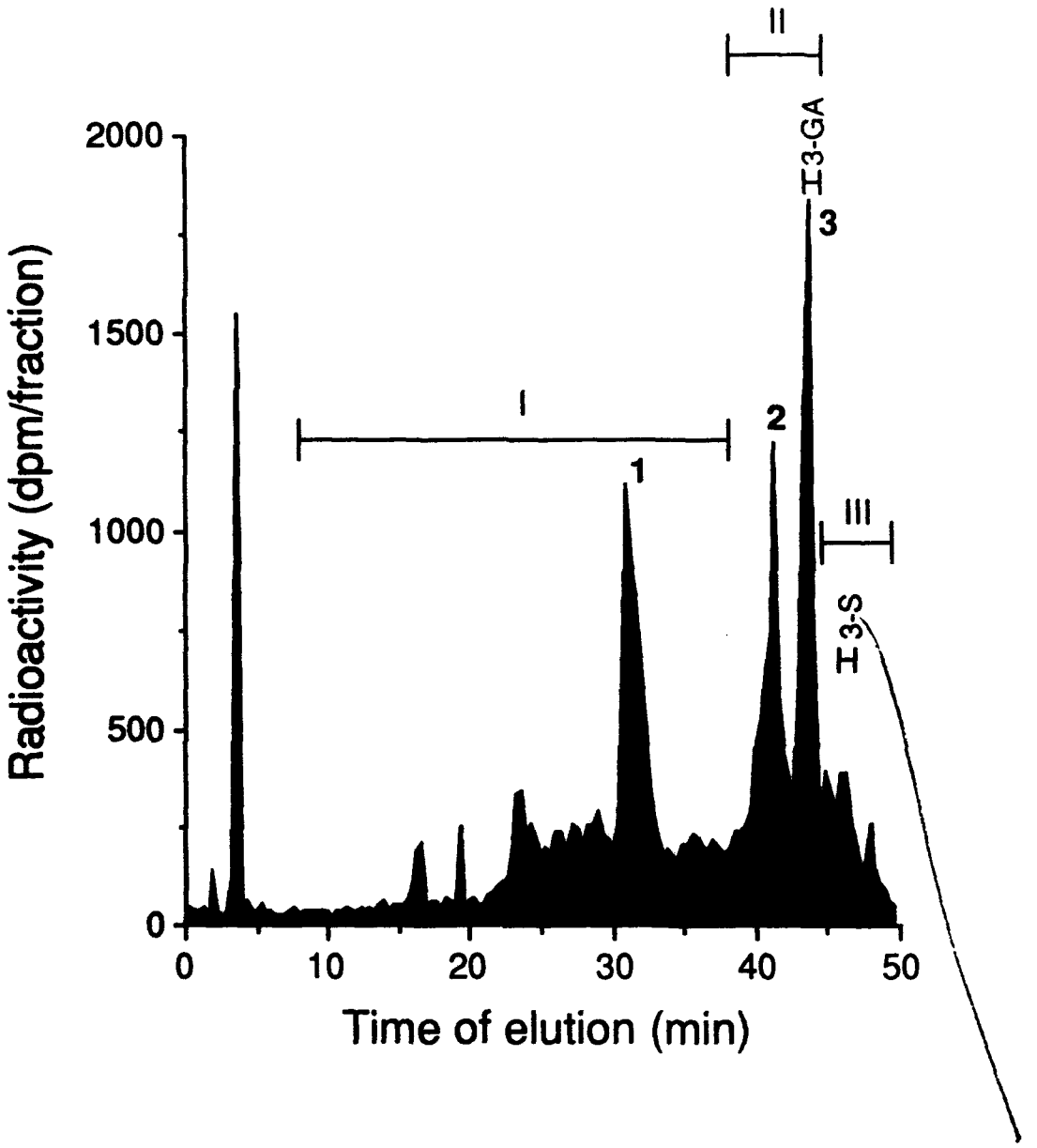

Radioactivity (dpm/fraction)
2000
1500
1000
500
0
0
10
20
30
40
50
Time of elution (min)
I
II
III
1
2
3
I3-GA
I3-S

Figure 10

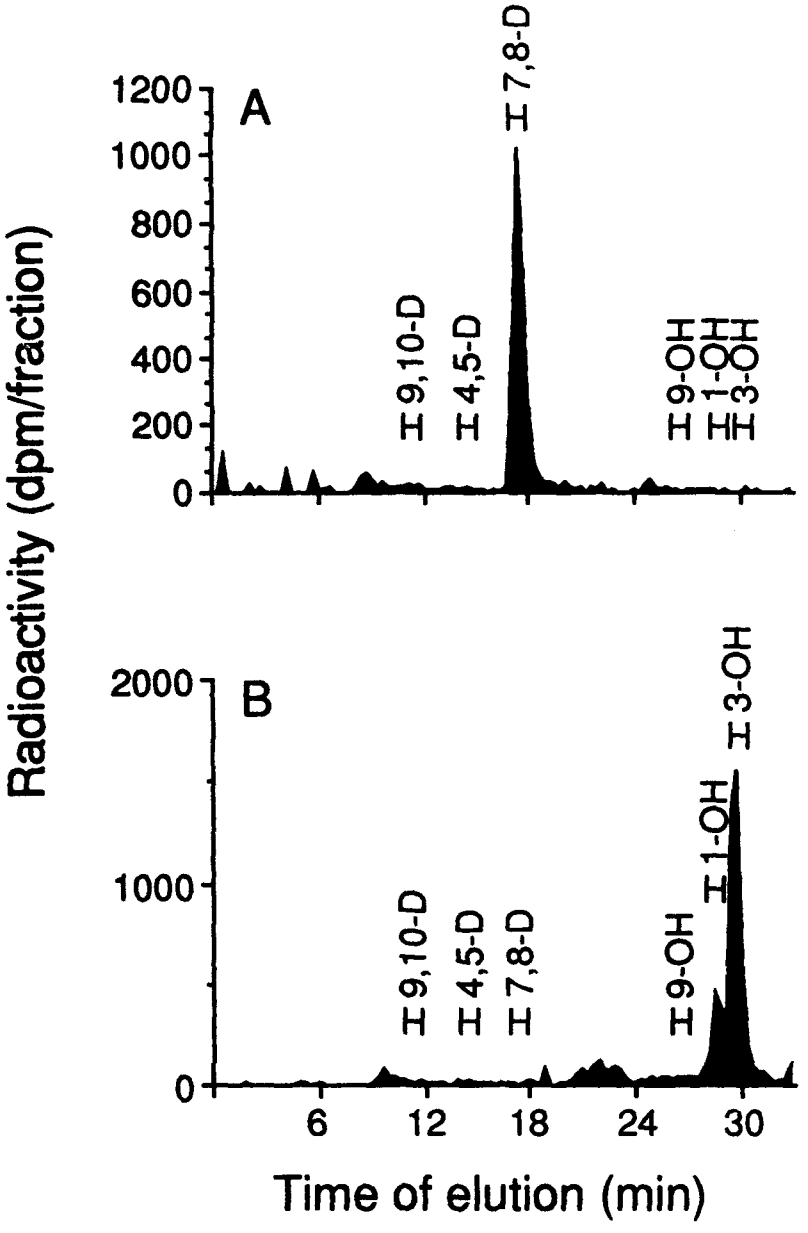

A
B
1200
1000
800
600
400
200
0
2000
1000
0
9,10-D
4,5-D
7,8-D
9-OH
1-OH
3-OH
6
12
18
24
30
Radioactivity (dpm/fraction)
Time of elution (min)

Figure 11

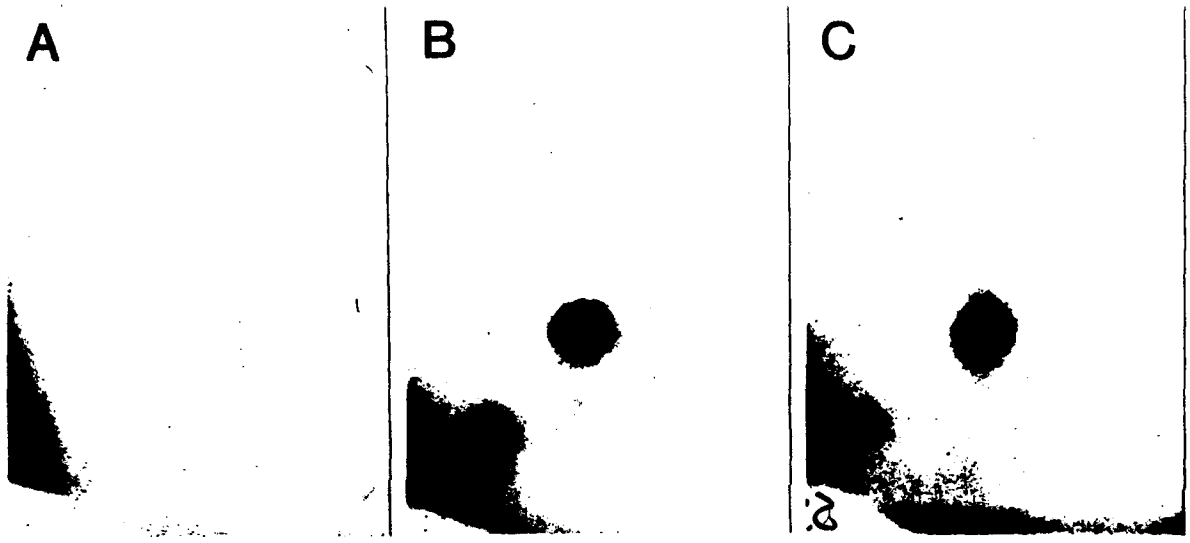


Figure 12

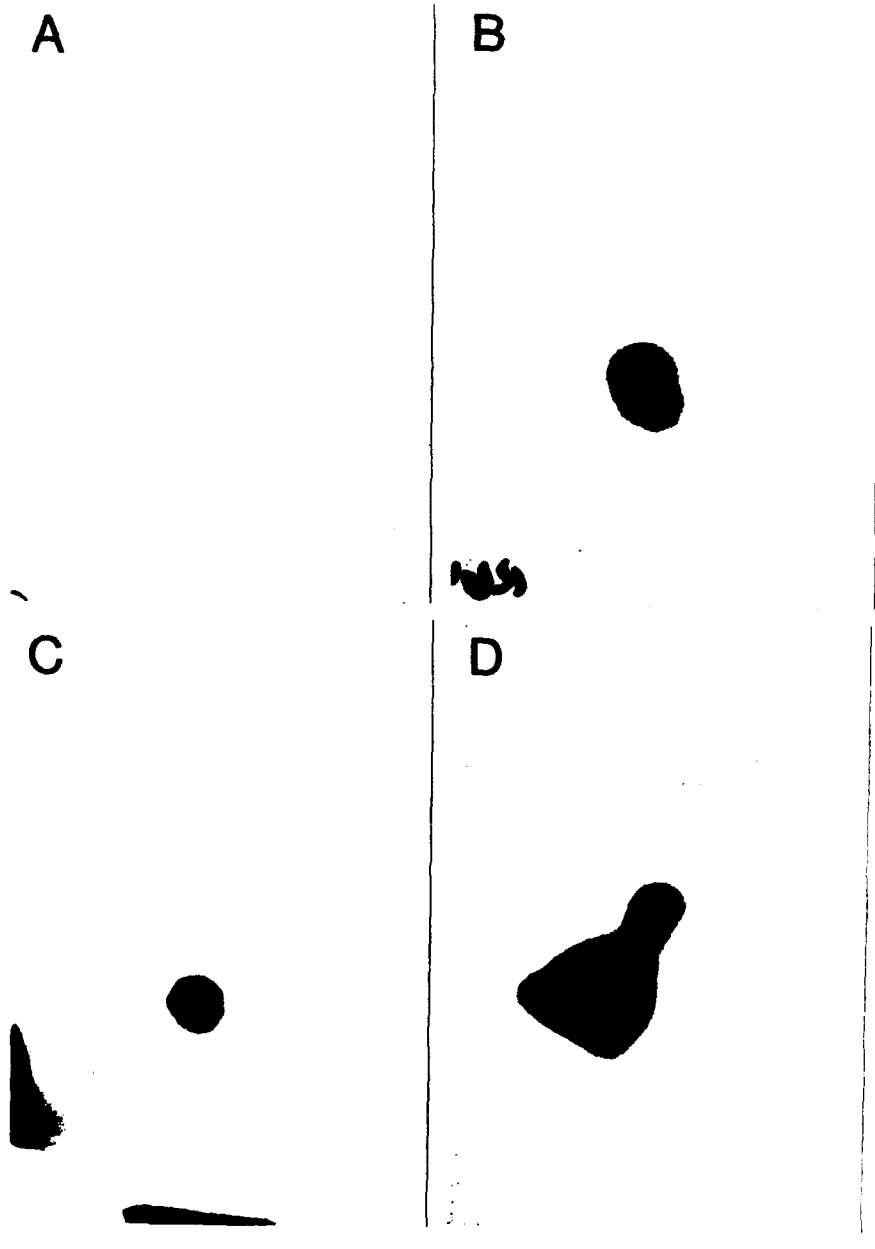


Figure 13

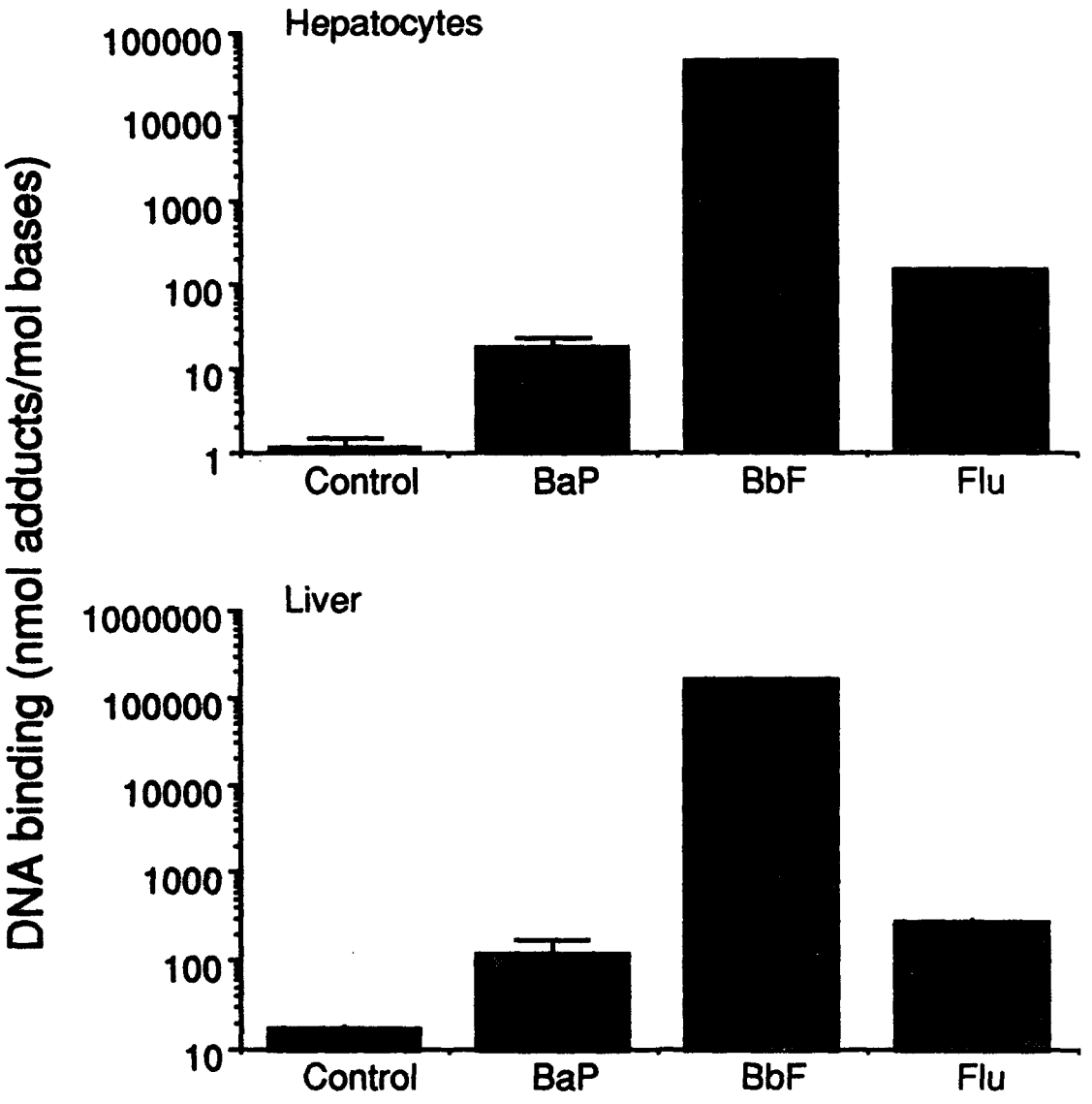

Hepatocytes
100000
10000
1000
100
10
1
Control
BaP
BbF
Flu
Liver
1000000
100000
10000
1000
100
10
Control
BaP
BbF
Flu
DNA binding (nmol adducts/mol bases)

Figure 14

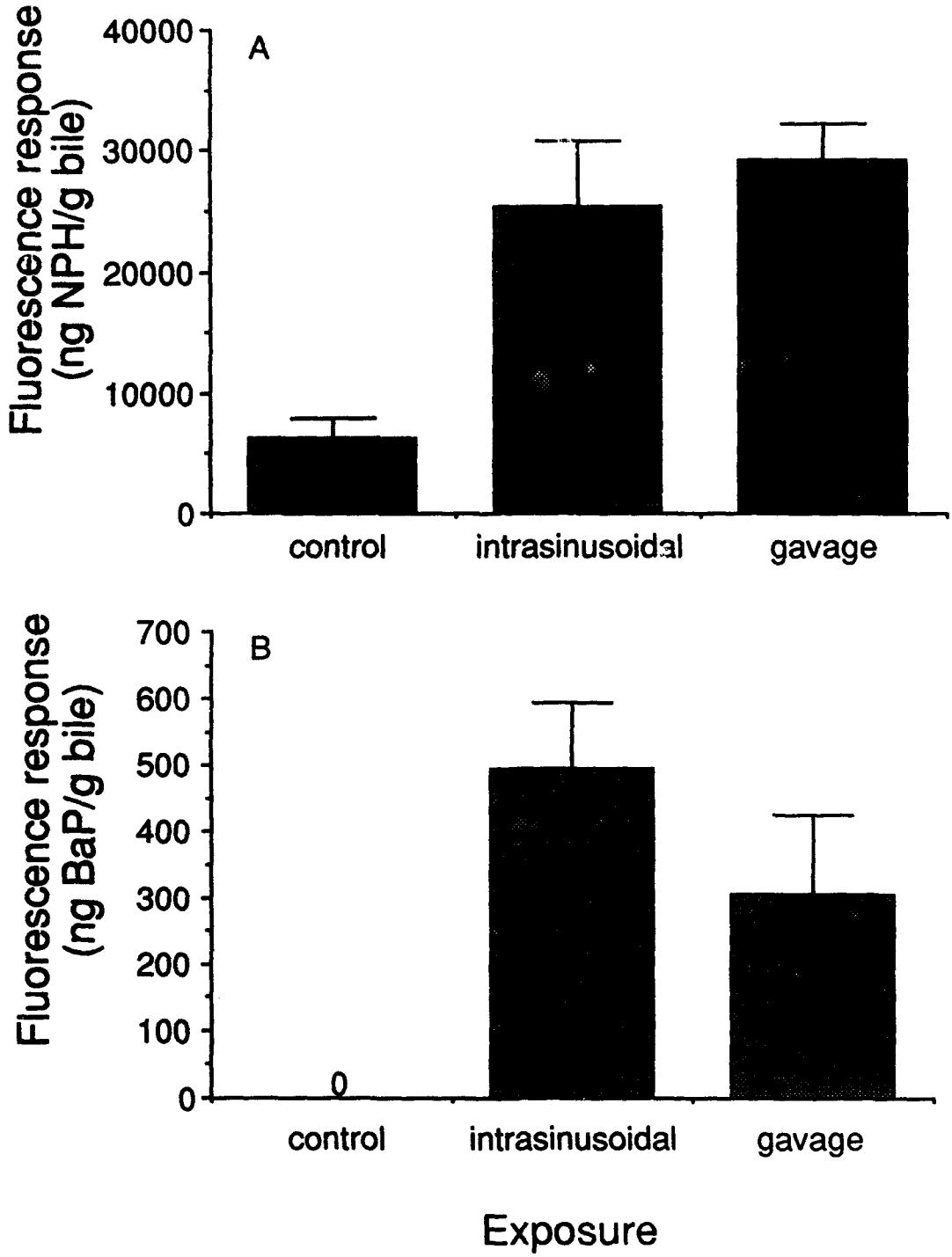

A
Fluorescence response (ng NPH/g bile)
40000
30000
20000
10000
0
control
intrasinusoidal
gavage
B
Fluorescence response (ng BaP/g bile)
700
600
500
400
300
200
100
0
0
control
intrasinusoidal
gavage
Exposure

Figure 15

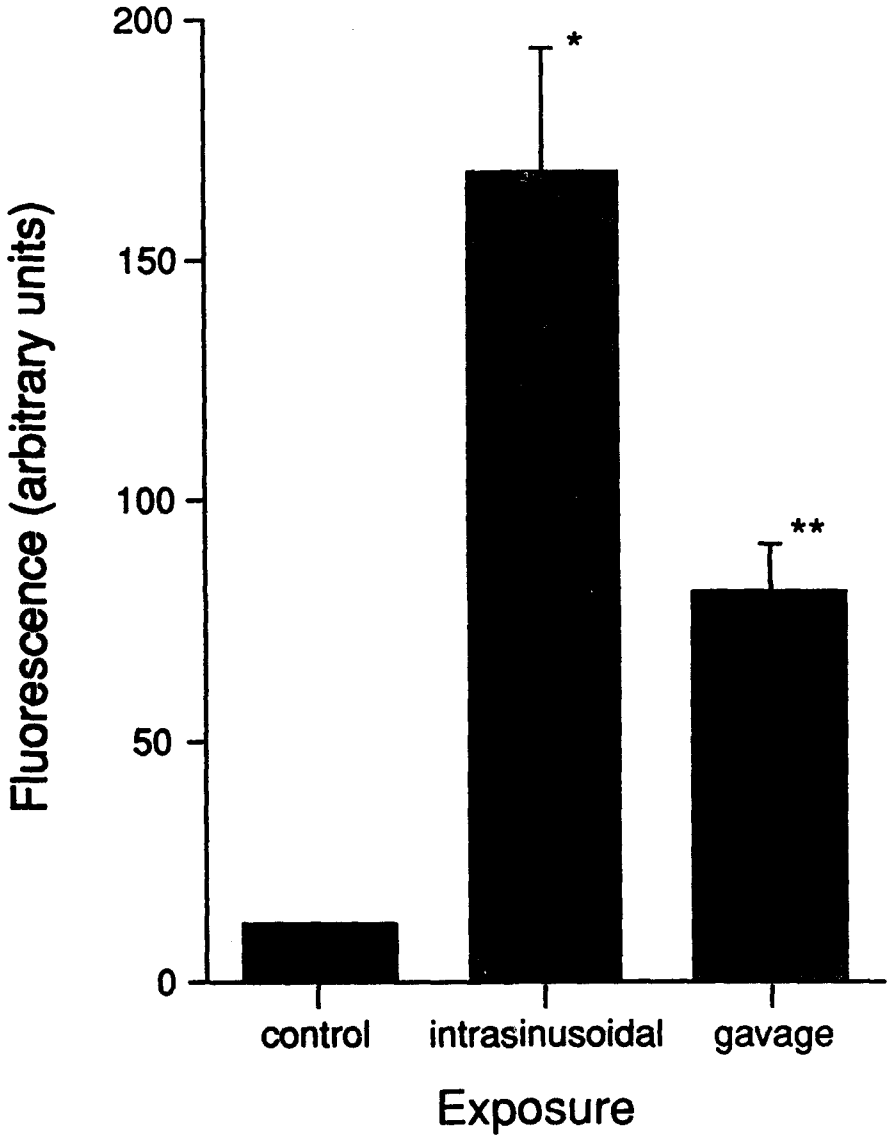

200
150
100
50
0
Fluorescence (arbitrary units)
*
**
control
intrasinusoidal
gavage
Exposure

Figure 16

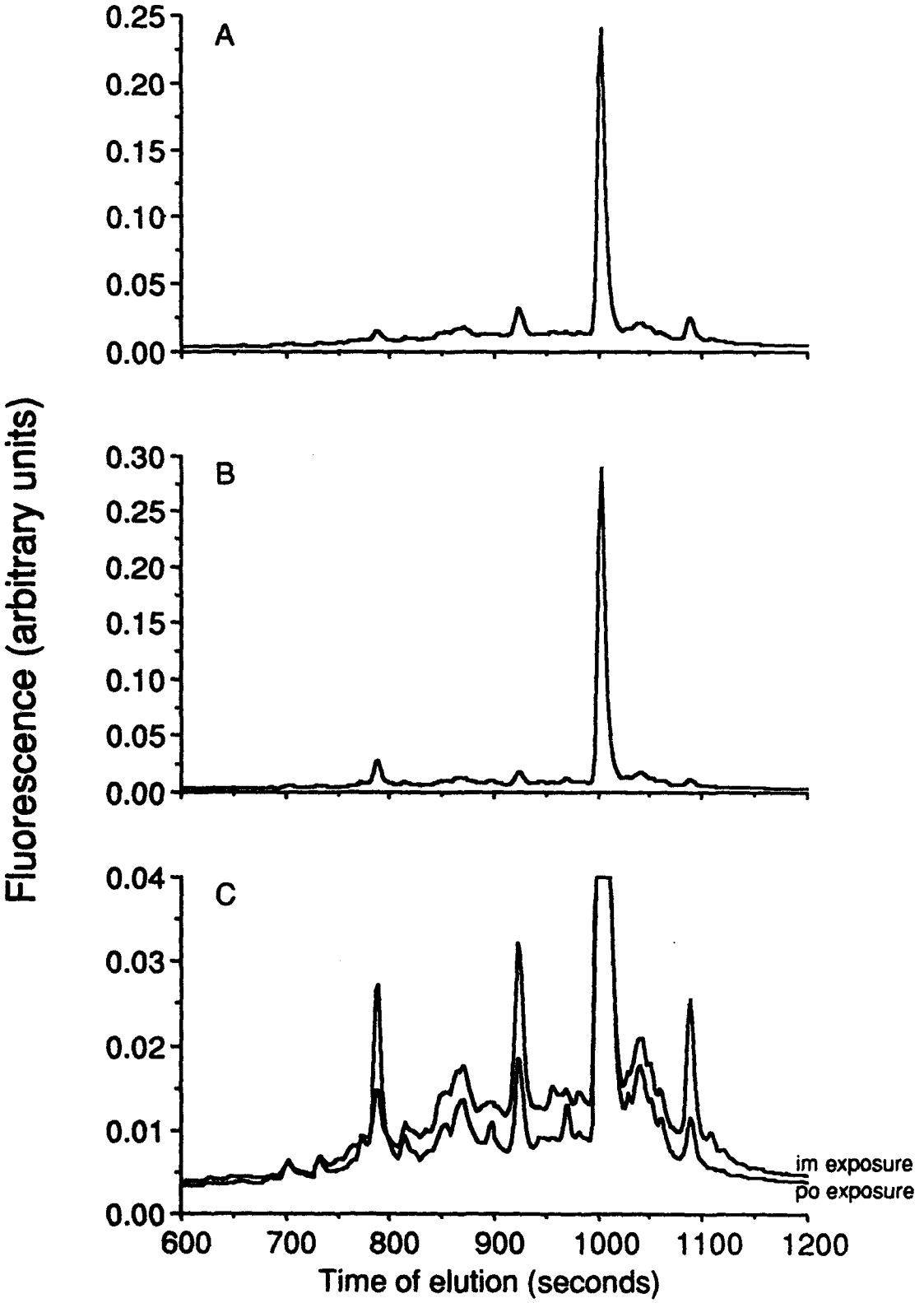
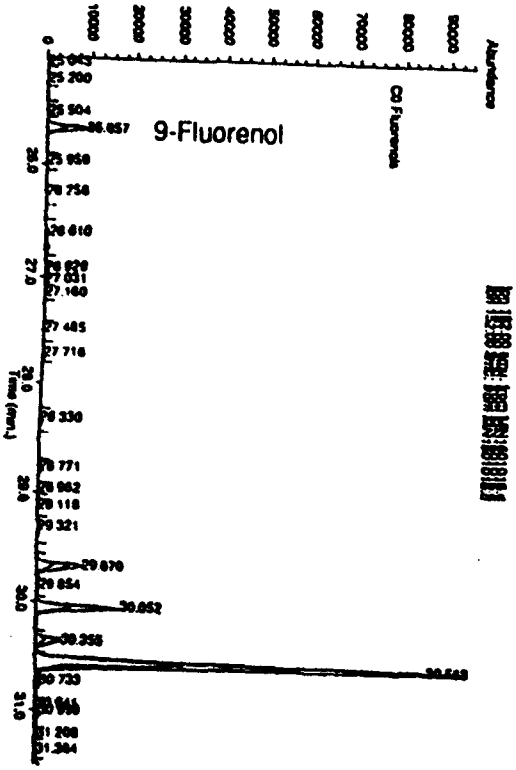
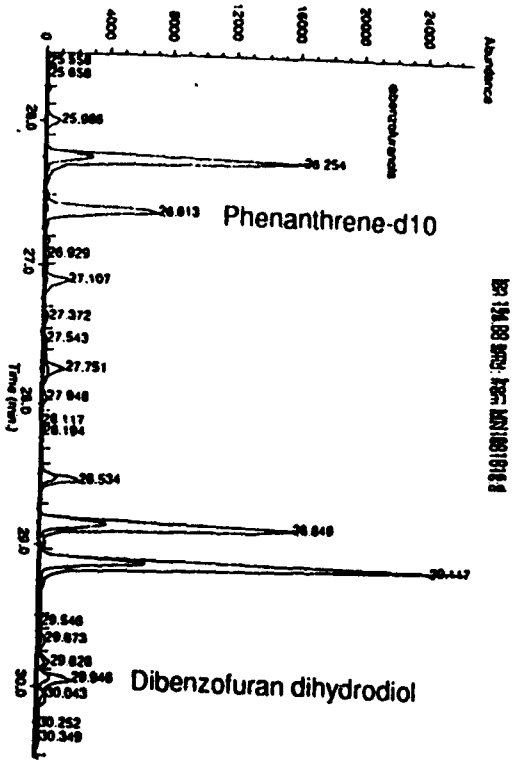
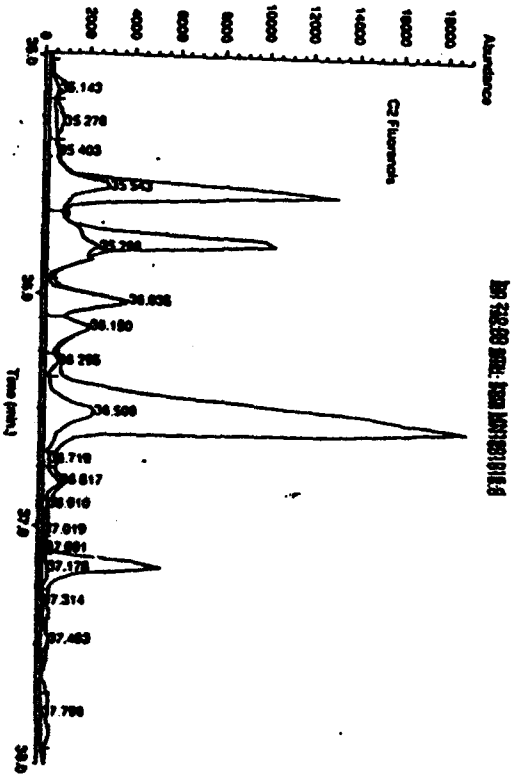

A
B
C
0.25
0.20
0.15
0.10
0.05
0.00
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.04
0.03
0.02
0.01
0.00
im exposure
po exposure
600
700
800
900
1000
1100
1200
Time of elution (seconds)
Fluorescence (arbitrary units)

Figure 17







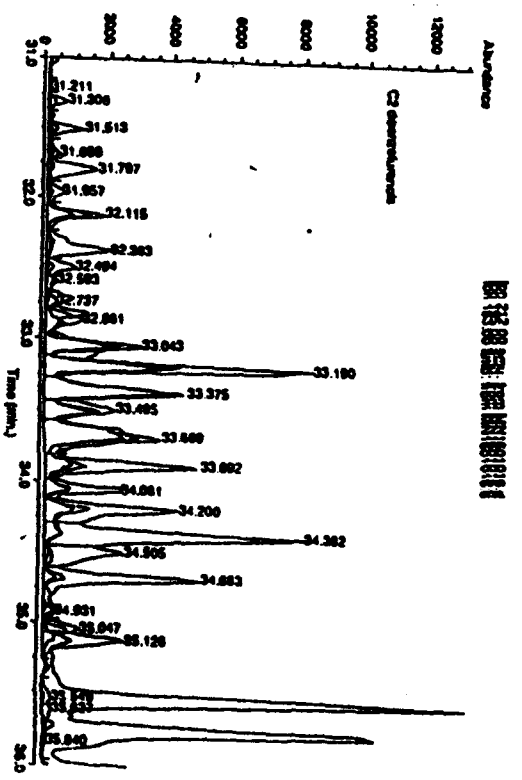
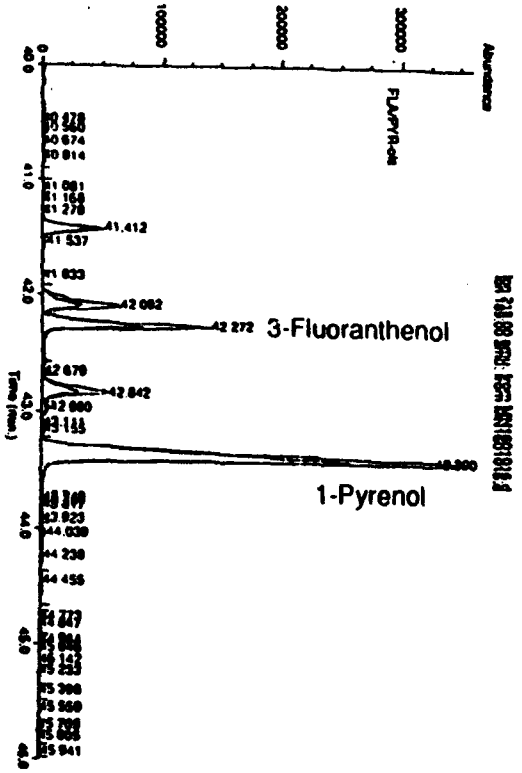
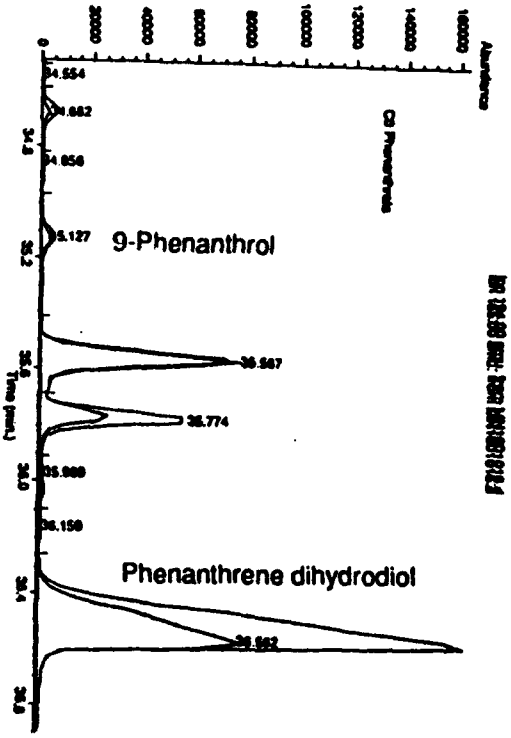
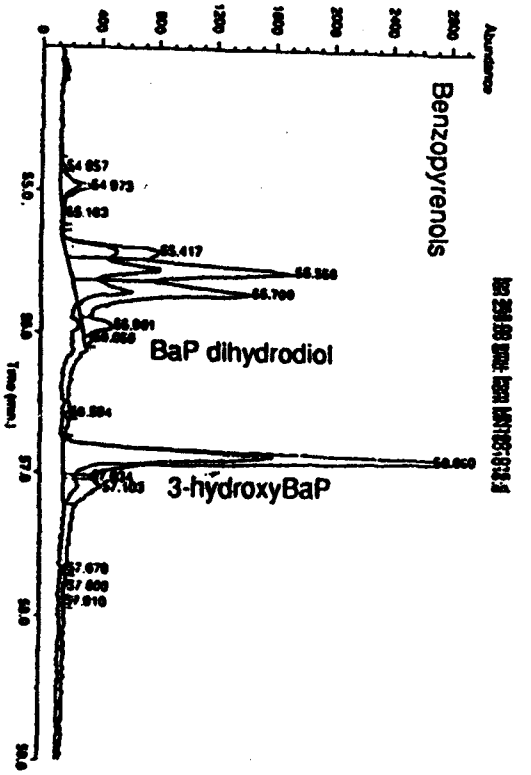


Figure 18


3-Fluoranthenol
1-Pyrenol


9-Phenanthrol
Phenanthrene dihydrodiol


Benzopyrenols
BaP dihydrodiol
3-hydroxyBaP

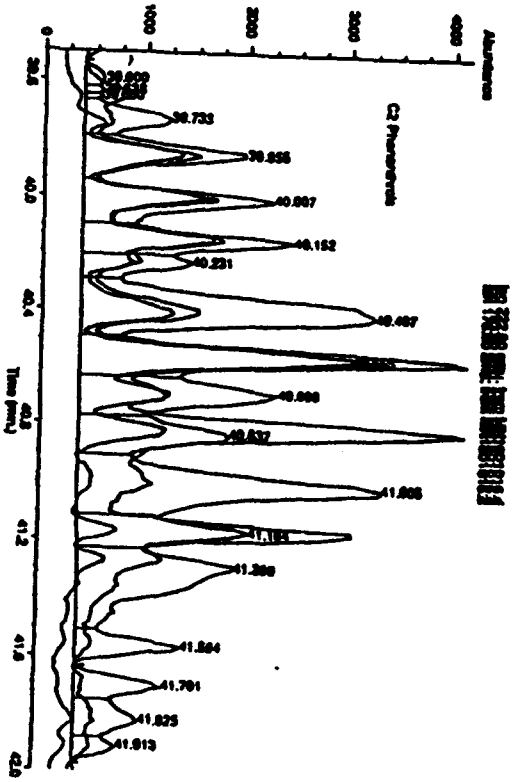


Figure 19

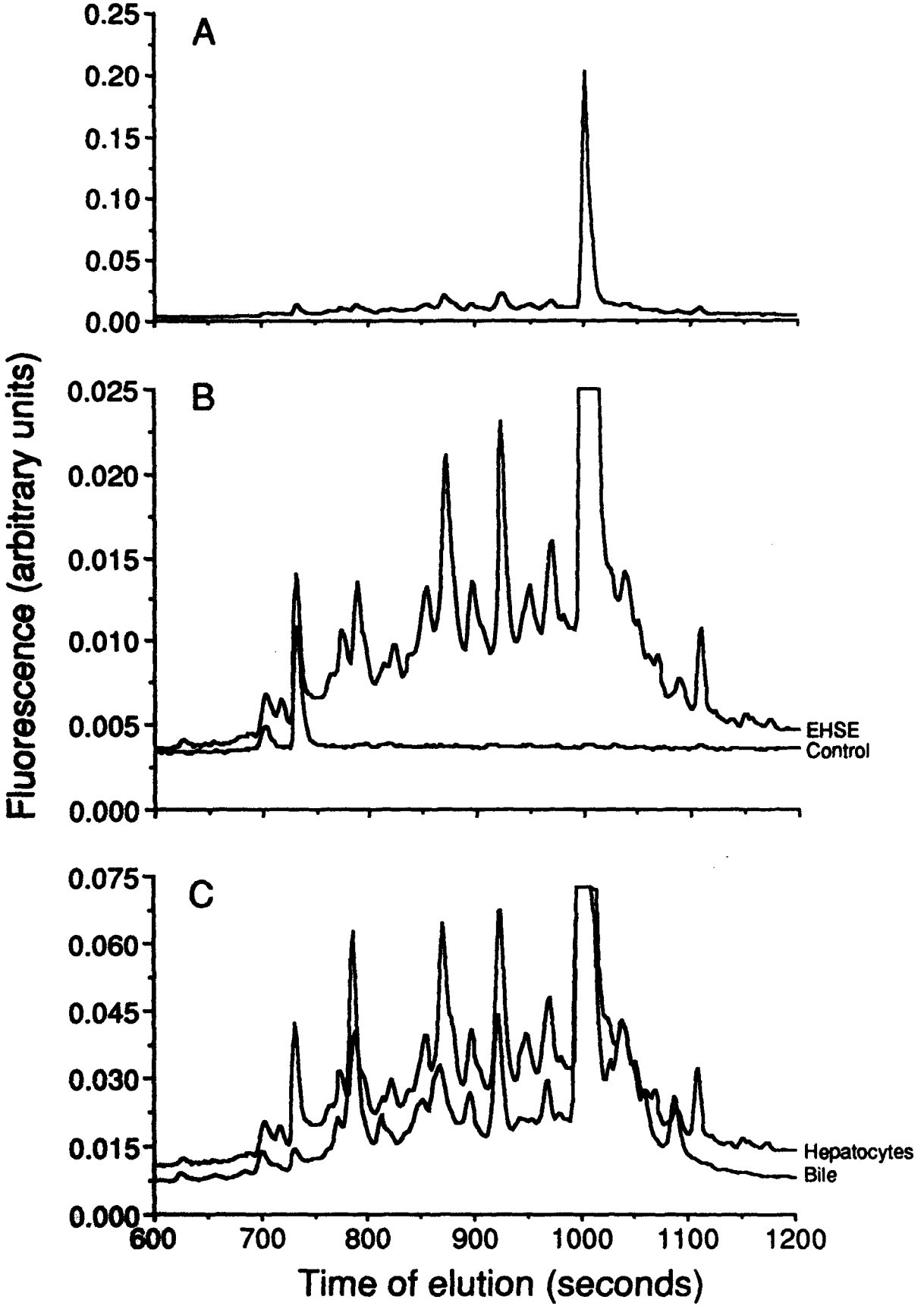

A
B
C
0.25
0.20
0.15
0.10
0.05
0.00
0.025
0.020
0.015
0.010
0.005
0.000
0.075
0.060
0.045
0.030
0.015
0.000
EHSE
Control
Hepatocytes
Bile
600
700
800
900
1000
1100
1200
Fluorescence (arbitrary units)
Time of elution (seconds)

Figure 20

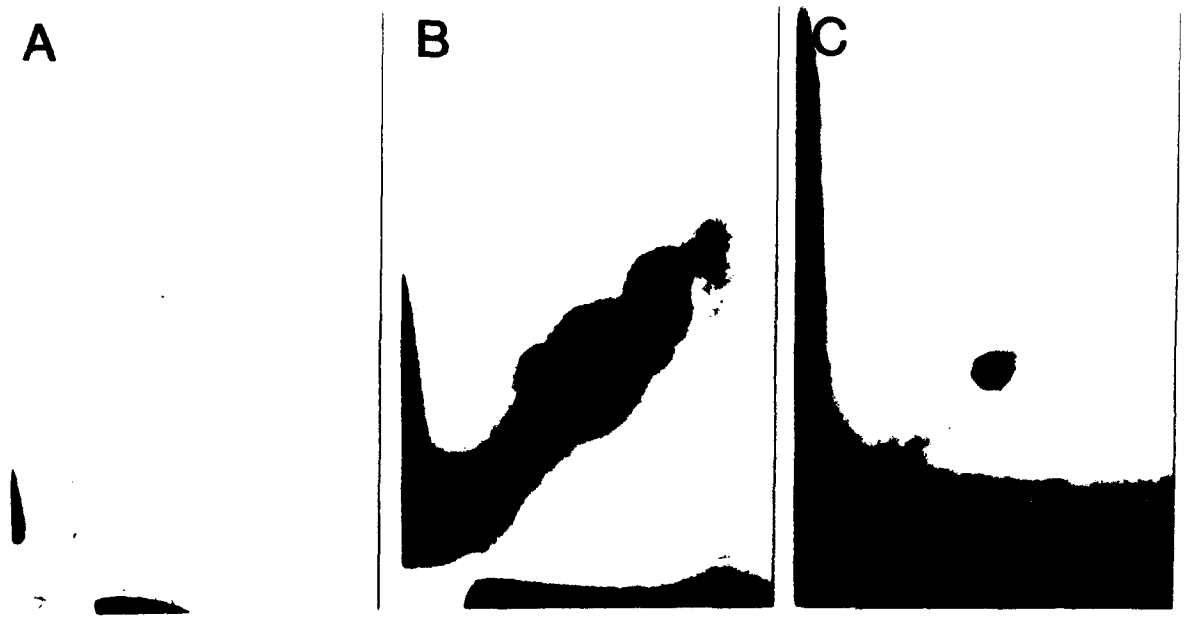


Figure 21

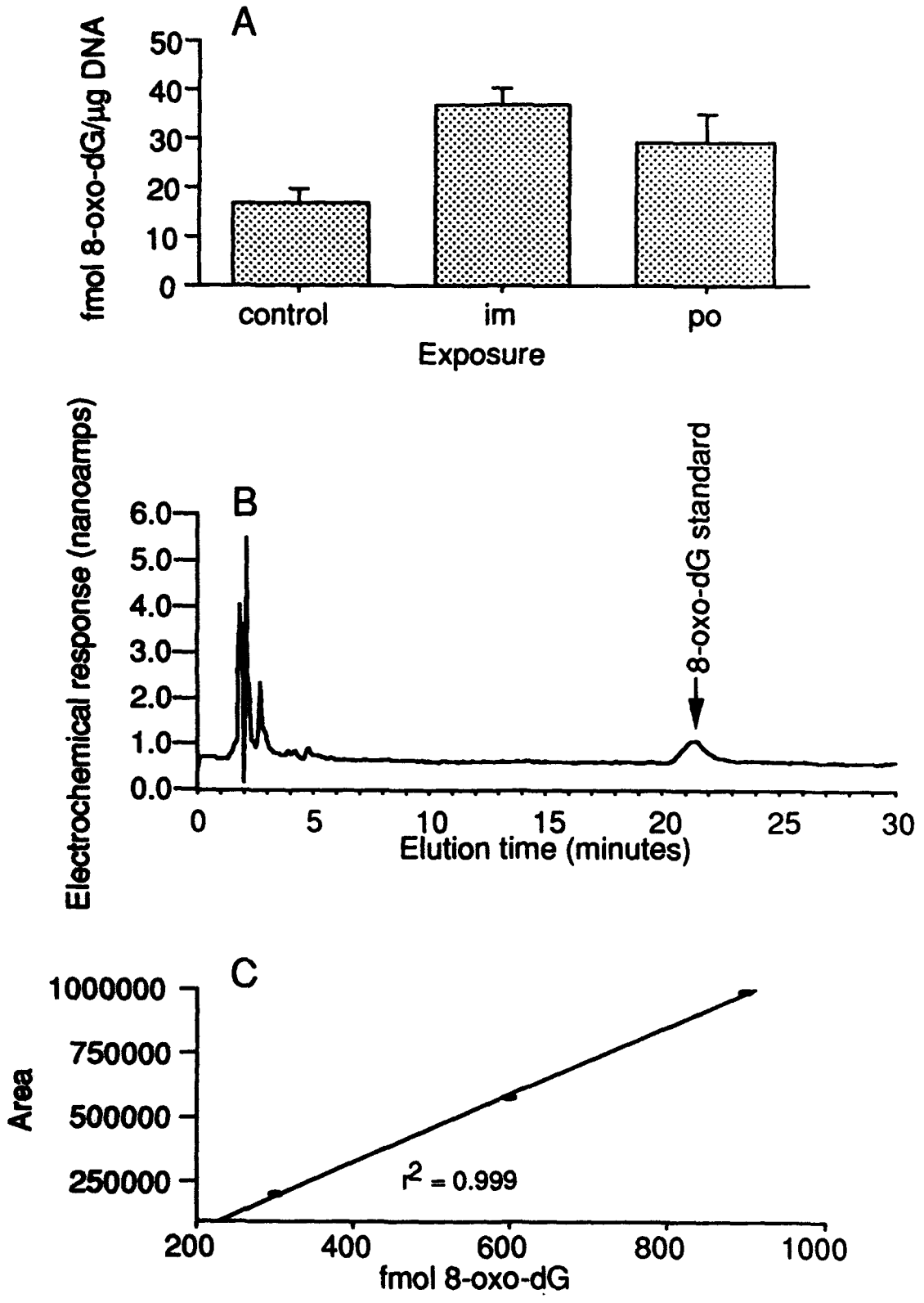

A
fmol 8-oxo-dG/µg DNA
50
40
30
20
10
0
control
im
po
Exposure
B
Electrochemical response (nanoamps)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
8-oxo-dG standard
0
5
10
15
20
25
30
Elution time (minutes)
C
Area
1000000
750000
500000
250000
r^2 = 0.999
200
400
600
800
1000
fmol 8-oxo-dG

Figure 22

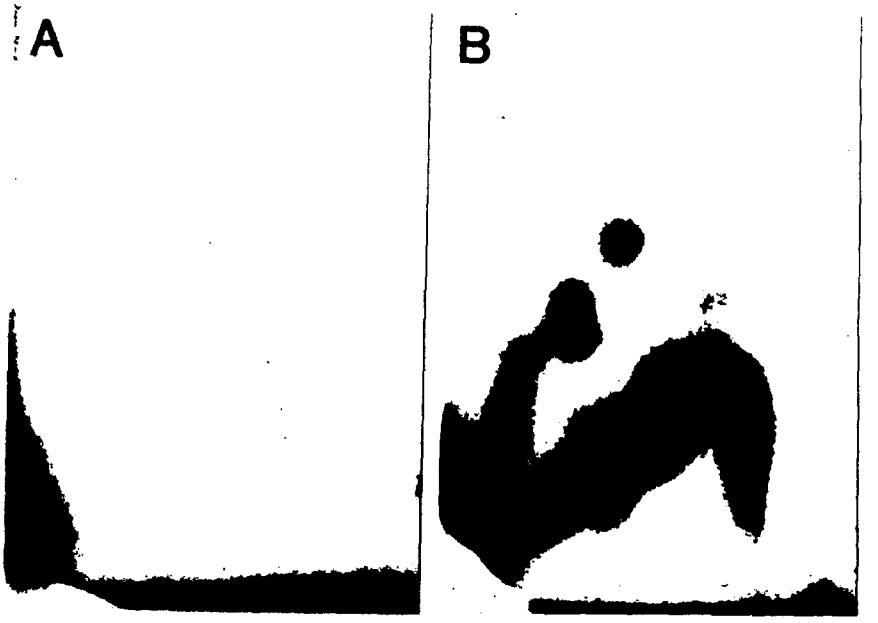


Figure 23

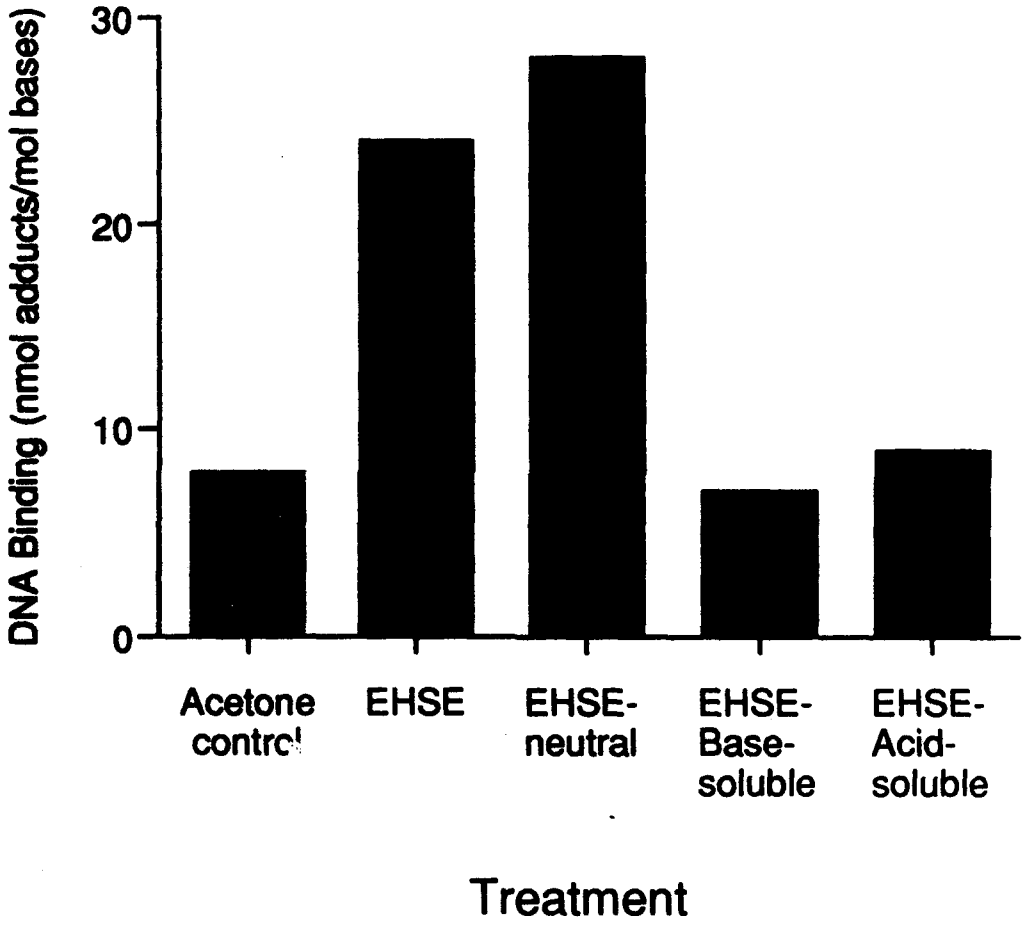

DNA Binding (nmol adducts/mol bases)
30
20
10
0
Acetone control
EHSE
EHSE-neutral
EHSE-Base-soluble
EHSE-Acid-soluble
Treatment

Figure 24